Structural Changes in Mitochondria Induced by Uncoupling Reagents

THE RESPONSE TO PROTEOLYTIC ENZYMES

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Interaction of uncoupling reagents with bovine serum albumin markedly inhibited its hydrolysis by proteolytic enzymes. The inhibition presumably is due to conformational transitions in the protein substrate induced by the binding of the ligand-uncoupling reagents. The proteolysis of casein, a protein that does not bind these reagents, was not affected, indicating that the proteinases themselves were not inactivated. In contrast, interaction of uncoupling reagents with freshly isolated rat liver mitochondria enhanced their susceptibility to proteolytic enzymes. This was shown by an increase in the release of ninhydrin-reacting material, by an increase in free acid groups and by a decrease in the turbidity of the mitochondrial suspensions. These effects, although opposite in direction to those obtained with albumin, are also presumed to indicate structural changes in the mitochondrial proteins and a disorganization of the protein-phospholipid complex. It is suggested that such structural alterations are expressed functionally as the uncoupling of oxidative phosphorylation.

Elsewhere we have presented evidence that led us to conclude that many reagents, particularly the substituted phenols, that uncouple oxidative phosphorylation in isolated rat liver mitochondria, interact with the protein moiety of the organelles (Weinbach & Garbus, 1964, 1965). We have further suggested that the uncoupling phenomenon is a consequence of conformational changes in the protein constituents of mitochondria induced by the protein-phenol interaction. In this report we present evidence that supports the suggestion that structural alterations occur in mitochondria as a consequence of their reaction with uncoupling reagents. Such changes are inferred from the altered response of mitochondria treated with these reagents to proteolytic enzymes. A preliminary account of this work has been presented (Garbus & Weinbach, 1966).

MATERIALS AND METHODS

Mitochondria. These were isolated from rat liver in 0.25 M-sucrose as described by Weinbach (1961a). Final suspensions were adjusted to contain 20 mg. of protein/ml.

Analytical. Proteolysis was determined colorimetrically with ninhydrin as described by Fasold & Gundlach (1963).

Rates of protein hydrolysis were also measured by electro-metric titration with a Radiometer Titrigraph (type SBR2c). Purified soluble proteins, or mitochondria suspended in 0.25 M-sucrose, were maintained at pH 8.5 by the addition of 0.05 N-KOH from a micro-syringe burette. Hydrolysis was initiated by the addition of the appropriate enzymes, and the rate of alkali addition was followed for at least 12 min. after the establishment of a linear rate.

Changes in the turbidity of mitochondrial suspensions were determined at 520 m μ with a Cary model 15 spectrophotometer. Initial extinctions were obtained with a neutral density screen of E 1.15 placed in the reference beam. Optical cells of 1.0 cm. light-path were used.

Protein was determined by the method of Gornall, Bardawill & David (1949).

Materials. Casein and bovine serum albumin (four-times crystallized) were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Casein was extracted with acetic acid-iso-octane to remove fatty acids according to the procedure of Goodman (1957). Albumin was freed of lipid by the method of Williams & Foster (1959). Carbonyl cyanide *m*-chlorophenylhydrazone was a gift from Dr P. G. Heytler, E. I. du Pont de Nemours and Co., Wilmington, Del, U.S.A., and 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole a gift from Dr R. B. Beechey of Shell Ltd., Sittingbourne, Kent. All other materials were of the highest purity commercially available.

Enzymes. Purified trypsin, a product of the Wilson Meat

Packing Co., Chicago, Ill., U.S.A. (lot no. 744367), was purchased from the Fisher Chemical Co., Silver Spring, Md., U.S.A. This product is a purified pancreatic extract with a tryptic activity of 3 National Formulary units/mg. and with slight lipase and diastase activities. Its intrinsic Ca^{2+} content of 0.5 $\mu\text{g.}/\text{mg.}$ obviated the requirement for additional Ca^{2+} in the hydrolysis experiments.

Pronase (a proteinase of *Streptomyces griseus*) was purchased from Calbiochem, Los Angeles, Calif., U.S.A. The enzyme solutions were freshly prepared for each experiment, were adjusted to pH 8.5, and were always kept cold before use.

RESULTS

Experiments with serum albumin. Markus (1965) and Markus, McClintock & Castellani (1966) have reported that the binding of low-molecular-weight ligands to bovine serum albumin greatly retards the proteolysis of this protein. We began this study by repeating the experiments of Markus and co-workers with serum albumin, with uncoupling reagents as the ligands. From Table 1, it is clear

that the binding of increasing molar ratios of pentachlorophenol increasingly inhibited the tryptic hydrolysis of bovine serum albumin. Results with casein as substrate showed that the tryptic hydrolysis of a protein that does not bind the uncoupling reagent under these conditions is not inhibited, and thus that the reagent does not act directly on the proteolytic enzyme. Consequently, the inhibition of the tryptic hydrolysis of albumin is a result of the interaction of the phenol with the protein substrate.

The extent of the inhibition indicates that a conformational change in the protein had been induced, and not the masking of the sites of tryptic attack. For example, there was 25% less proteolysis with the binding of the equivalent of 1 mole of pentachlorophenol/30–40 moles of susceptible bonds of serum albumin.

The inhibition was not unique for pentachlorophenol, nor, as Rossini (1963) had shown, for 2,4-dinitrophenol. The ability to inhibit proteolysis of serum albumin is a property of many reagents,

Table 1. *Effect of reagents on the proteolysis of albumin*

A solution of bovine serum albumin (21.7 mg./ml.) was adjusted to pH 8.5 with KOH. Of this solution, 0.8 ml. was added to 7.2 ml. of 0.25 M-sucrose contained in the vessel of the pH-stat. Trypsin (0.4 ml. of a 0.42% solution previously adjusted to pH 8.5) was added to initiate hydrolysis, the pH was maintained at 8.5 by the addition of 0.05 N-KOH, and the temperature was kept at 25°. The experiment was repeated with each of the compounds listed. These were added in small volumes of concentrated solutions to give the desired final concentrations. The antibiotic reagents, dicoumarol, rotenone and Amytal, were added as ethanolic solutions. It was ascertained that the small amount of ethanol added has no effect on proteolysis.

Compound	Concn. (mm)	Molar ratio ([Reagent]/[albumin])	Inhibition of proteolysis (%)
Pentachlorophenol	0.015	0.5:1	25
Pentachlorophenol	0.03	1:1	55
Pentachlorophenol	0.06	2:1	77
Pentachlorophenol	0.09	3:1	85
Pentachlorophenol	0.12	4:1	86
Pentachlorophenol	0.15	5:1	85
2,4-Dinitrophenol	0.03	1:1	25
2,4-Dinitrophenol	0.06	2:1	50
2,4-Dinitrophenol	0.09	3:1	60
2,4,6-Trinitrophenol	0.03	1:1	25
2,4,6-Trinitrophenol	0.06	2:1	60
Carbonyl cyanide <i>m</i> -chlorophenylhydrazone	0.03	1:1	19
Carbonyl cyanide <i>m</i> -chlorophenylhydrazone	0.06	2:1	50
4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole	0.03	1:1	46
4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole	0.06	2:1	62
4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole	0.09	3:1	66
Dicoumarol	0.03	1:1	60
Dicoumarol	0.06	2:1	80
Dicoumarol	0.12	4:1	75
CaCl_2	1.0	33:1	22
CaCl_2	10.0	333:1	85
Gramicidin	0.3	10:1	0
Rotenone	0.3	10:1	0
Amytal	0.3	10:1	0
Oligomycin	10 $\mu\text{g.}/\text{mg.}$ of albumin		0
Antimycin A	10 $\mu\text{g.}/\text{mg.}$ of albumin		0

including all the true uncoupling agents tested (Table 1). Oligomycin, antimycin A, gramicidin, rotenone and Amytal, even at high concentrations, were without significant effect. The possibility that albumin does not bind the antibiotic reagents and rotenone is suggested by the fact that it fails to counteract the effects of these reagents on the functional activity of mitochondria (Weinbach & Garbus, 1966*a,b*). The reagents listed in Table 1 also had no effect on the proteolysis of casein.

Other experiments demonstrated that the proteolysis of serum albumin catalysed by Pronase was also inhibited by those reagents effective with trypsin. This bacterial proteinase presumably attacks peptide bonds differing from those hydrolysed by trypsin (Hagihara, 1960).

Experiments with mitochondria. Having established the inhibition of the proteolysis of albumin, we tested the effects of the uncoupling reagents on the proteolytic digestion of an isolated mitochondrial protein and of intact mitochondria. The tryptic hydrolysis of an insoluble mitochondrial protein with properties similar to that of structural protein (Weinbach & Garbus, 1965) was inhibited 20% after its reaction with pentachlorophenol (4mM). In contrast, freshly isolated intact rat liver mitochondria, previously treated with pentachlorophenol and then exposed to Pronase, exhibited an enhancement rather than inhibition of proteolysis. This effect, which was apparent with uncoupling concentrations of the halophenol (40 μ M), was especially prominent with higher concentrations (Fig. 1). Although this enhancement was unexpected in view of the results of the experiments with albumin, a change in susceptibility to proteolytic digestion has been considered as evidence of a conformational transition in protein substrates. The increased susceptibility of denatured proteins to proteolysis is a well-known example (Putnam, 1953).

When analogous experiments were done with trypsin or other proteolytic enzymes, or with 2,4-dinitrophenol, an enhanced rate of hydrolysis also was observed.

Turbidimetric assay of proteolysis. We also utilized the rapid clearing of the turbidity of the mitochondrial suspension undergoing tryptic hydrolysis as a convenient indication of changes in the susceptibility to proteolysis.

As illustrated in Fig. 2, the turbidity of a mitochondrial suspension exhibited a sudden and rapid decrease in extinction approx. 12min. after the addition of trypsin.

To facilitate the hydrolysis of mitochondria with trypsin, preincubation at pH 8.5 was desirable. As discussed below, this resulted in partial uncoupling, presumably associated with altered mitochondrial structure. When a minimal uncoupling concentra-

Fig. 1. Effect of pentachlorophenol on the proteolysis of mitochondria. Mitochondria (50 mg. of protein) were preincubated in 5 ml. of 0.04M-tris buffer, pH 8.5, with pentachlorophenol (O, 4mM; ●, 0.4mM) and without pentachlorophenol (Δ). After preincubation for 10 min. at 30°, 5 ml. of 0.1% Pronase was added to initiate hydrolysis. At the times shown, 1.0 ml. samples were removed for analysis by the ninhydrin procedure of Fasold & Gundlach (1963). The nature of the ninhydrin-reacting material was not further characterized.

Fig. 2. Effect of pentachlorophenol on the turbidity of mitochondrial suspensions exposed to trypsin. Mitochondria (8 mg. of protein) were suspended in 4 ml. of 0.25M-sucrose containing 25mM-tris, pH 8.5. Another vessel contained the same components plus 20 μ M-pentachlorophenol (PCP). After 10 min. preincubation at 25°, 0.2 ml. of 0.42% trypsin was added to each of the vessels and the contents were mixed by gentle inversion and then transferred to the optical cells of the recording spectrophotometer. The extinction of each of the two suspensions was alternately determined at frequent intervals.

tion of pentachlorophenol (20 μ M) was present, the mitochondria were even more susceptible to the tryptic attack, as if the effect of the uncoupling reagent was additive to the effect of the preincubation. Under these conditions the hydrolysis was immediately apparent upon the addition of trypsin, with the rapid decrease occurring considerably earlier than that observed with the control preparation.

Mitochondria treated with pentachlorophenol at 0° and then washed free of the unbound phenol (Weinbach & Garbus, 1966a) were also more susceptible to proteolysis. Pentachlorophenol at minimal uncoupling concentrations in the absence of trypsin had no effect on the turbidity of the mitochondrial suspension, showing that the enhancement of tryptic hydrolysis was not a consequence of gross swelling of mitochondria.

Experiments with 2,4-dinitrophenol showed that it had no intrinsic effect on the swelling of mitochondria (Lehninger, 1962), but that it did elicit the rapid onset of the decrease in turbidity of the mitochondrial suspension subsequently exposed to trypsin.

Significantly, 2,4,6-trinitrophenol, a substituted phenol that is devoid of uncoupling properties (cf. Weinbach, 1954), had little effect on the tryptic hydrolysis of mitochondria whereas, in the same experiment, 2,4-dinitrophenol enhanced proteolysis. Both phenols are bound to mitochondria though presumably at different sites (Weinbach & Garbus, 1965). It is noteworthy, however, that the interaction with 2,4,6-trinitrophenol did inhibit the tryptic hydrolysis of albumin (Table 1).

Changes in the susceptibility of mitochondria toward proteolysis are not restricted to the action of uncoupling phenols. The uncoupling reagents carbonyl cyanide *m*-chlorophenylhydrazine (Heytler, 1963) and 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (Beechey, 1966) were also effective in eliciting the rapid response to tryptic hydrolysis. Evidence of increased susceptibility was observed with concentrations of the latter compound as low as 1 μ M.

Effect of aging. Mitochondria used 6 hr. after their isolation were more susceptible to the action of trypsin than were fresh preparations. The rapid clearing began at approx. 8 min., instead of the 10–12 min. usually seen with freshly isolated mitochondria. Other experiments clearly showed that aging the mitochondria increased their susceptibility to tryptic attack. Preparations stored at 4° for 24 or 48 hr. showed an immediate decrease in extinction on the addition of trypsin. Reaction of these stored preparations with pentachlorophenol, or with 2,4-dinitrophenol, either had little further effect or delayed their susceptibility to trypsin. Mitochondria that contained bound pentachlorophenol,

and that were digestible by trypsin when initially assayed, were much more resistant to proteolysis after storage for 24 hr. than similarly stored controls.

The influence of aging was particularly evident in experiments with gramicidin. Reaction of freshly isolated mitochondria with 5 μ M-gramicidin enhanced the susceptibility to tryptic hydrolysis in a manner similar to that seen with the other uncoupling reagents. Gramicidin, however, had little or no effect on mitochondria that had been stored for 4 hr. at 4°. With mitochondria that had been stored for 8 hr. or more, the action of gramicidin was to retard proteolysis.

Effect of other reagents. Ca²⁺ ions (1 mM) also promoted the rapid response of mitochondria to proteinases. Antimycin A (2 μ g./mg. of mitochondrial protein) accelerated, whereas oligomycin (2 μ g./mg. of mitochondrial protein) and rotenone (2 μ M) retarded the onset of the rapid decrease of turbidity. Other substances that inhibited the hydrolysis were ATP (3 mM), Mg²⁺ (5 mM) and EDTA (1 mM). Pancreatic ribonuclease and diastase were without effect either with control or with pentachlorophenol-treated mitochondria.

Correlation of changes in turbidity with release of acid. To test whether the changes in extinction of mitochondrial suspensions exposed to proteinases were related to proteolysis, measurements of extinction were correlated with the release of acid groups. Preliminary tests had shown that the proteolysis of mitochondria could be followed readily by electrometric titration of the liberated acid groups.

Four such experiments are summarized in Fig. 3. Expts. 1, 2 and 3 illustrate tryptic hydrolysis of freshly isolated mitochondria previously treated with each of four uncoupling reagents used in the turbidimetric assays. The arrows in Fig. 3 indicate the time of onset of the rapid decrease in turbidity as seen in the spectrophotometer. Clearing was also observed in the titration vessel, and was accompanied, in the controls, by an increased rate of alkali addition. On the other hand, the initial rate of alkali addition was always more rapid with the mitochondria previously treated with uncoupling reagents, indicating that an enhanced rate of proteolysis preceded the onset of the rapid decrease in turbidity. In experiments with Pronase (Fig. 3, Expt. 4) the rate of mitochondrial hydrolysis never changed abruptly during the time of observation, and although prior reaction with pentachlorophenol led to clearing of the suspensions, the dramatic effect seen with trypsin was never observed. These results may reflect either the influence of different sites of proteolytic attack by this enzyme, or of experimental conditions less favourable for its activity.

Reversibility experiments. As serum albumin can

Fig. 3. Effect of uncoupling reagents on the proteolysis of mitochondria. Proteolysis was determined by electrometric titration as described in the legend to Table 1, and in the text. Mitochondria (16 mg. of protein) were suspended in 8.0 ml. of 0.25 M-sucrose and the pH was adjusted to 8.5. After incubation at 25° for 10 min., 0.4 ml. of 0.42% trypsin was added in Expts. 1, 2 and 3. In Expt. 4, 0.4 ml. of 0.42% Pronase was used. The concentrations of the uncoupling reagents were: carbonyl cyanide *m*-chlorophenylhydrazone (CCP), 10 μ M; pentachlorophenol (PCP), 50 μ M; 2,4-dinitrophenol (DNP-OH), 0.5 mM; 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB), 10 μ M. The arrows indicate the onset of the rapid decrease in turbidity, as seen in companion experiments conducted with the spectrophotometer.

Fig. 4. Restoration with albumin of resistance to tryptic hydrolysis. (a) In the control experiment, mitochondria (16 mg. of protein), suspended in 8 ml. of 0.25 M-sucrose containing 25 mM-tris, pH 8.5, were incubated at 25° for 8 min. At this time, 4 ml. of the suspension was removed and 2 μ M-bovine serum albumin (BSA) was added. Two minutes later, 0.2 ml. of 0.42% trypsin was added to each suspension and their extinctions were determined. (b) In the companion pentachlorophenol experiment, the mitochondria were treated with 4 μ M-pentachlorophenol for 8 min. before the addition of albumin.

restore functional activity to mitochondria uncoupled by substituted phenols (Weinbach & Garbus, 1966a,b) it was ascertained whether this protein could restore resistance to proteolysis to mitochondria treated with these reagents. In the experiments shown in Fig. 4, mitochondria were incubated with trypsin in the presence or absence of albumin. With the control preparation, albumin delayed the onset of the rapid decrease in turbidity

by 25%. With mitochondria previously exposed to pentachlorophenol, albumin delayed the onset by 100%. It is evident that the effect of albumin was proportionately much greater with the mitochondria treated with the uncoupling reagent. However, the delay in the response to trypsin compared with that seen with the control preparations could not be restored. It should be pointed out that in these experiments it was found necessary to use

minimal amounts of albumin ($2\mu\text{M}$), because larger amounts (e.g. $10\mu\text{M}$) of this protein markedly delayed or completely suppressed the hydrolysis of mitochondria. The mechanism by which albumin protects mitochondria is unknown. Competition for the proteinase appears unlikely since casein had no effect on the tryptic hydrolysis of mitochondria.

Other attempts to effect reversal by removing the uncoupling reagents with procedures involving centrifugation, washing and resuspension were unsuccessful. These manipulations in themselves further altered the mitochondria to an extent that obviated any possible restoration. The results of these experiments again emphasize that the response to proteolysis is an extremely sensitive indication of the structural state of the mitochondrion.

Changes in functional activity. Additional tests were made with a polarographic technique (Weinbach & Garbus, 1966b) to determine the functional state of mitochondria incubated under the conditions used in the proteolytic experiments. Mitochondria preincubated in 0.25M -sucrose at pH 8.5 for 10 min. at 25° when tested with various substrates exhibited diminished respiratory control and were 'loosely coupled'. The addition of trypsin to such mitochondria led to an almost immediate loss of respiratory control and of coupled phosphorylation, followed by a less rapid inactivation of the enzymes that catalyse the oxidation of NAD-linked substrates. These functional alterations always occurred before the onset of the rapid decrease in turbidity induced by trypsin. However, the capacity to oxidize NADH and succinate by an antimycin A-sensitive pathway was retained by the clarified preparations for 20–30 min. after the addition of trypsin; NADH oxidase activity was more resistant. Addition of cytochrome *c* to the clarified preparations enhanced their ability to oxidize these two substrates, suggesting that loss of electron carriers preceded inactivation of the enzymes. The inactivation of succinate oxidase and cytochrome oxidase systems by treating mitochondria with trypsin has been described by Dianzani (1953).

DISCUSSION

The data presented in this paper show that interaction of uncoupling reagents with bovine serum albumin (Table 1) or with isolated rat liver mitochondria (Figs. 2 and 3) alters the susceptibility to proteolysis. In view of the work of Markus (1965) with other low-molecular-weight ligands, it is reasonable to assume that the inhibition of proteolysis of albumin is due to conformational changes in the protein substrate induced by the binding of the uncoupling reagents. Previous work in our Laboratory (Weinbach & Garbus, 1964, 1965) led

us to believe that uncoupling reagents interact with protein sites in mitochondria. If so, this interaction could alter the structural organization of mitochondrial protein. With freshly isolated mitochondria treated with uncoupling reagents, the enhanced response to proteolysis may reflect such a structural alteration; in aged mitochondria, however, the response to uncoupling agents is a retardation of proteolysis.

These differential effects may reflect the interaction of the uncoupling reagents with the membrane systems of mitochondria. That mitochondria are composed of two membrane systems differing in composition and function is now well established (Parsons, 1965; Parsons, Williams & Chance, 1966; Green & Tzagoloff, 1966; Bachmann, Allmann & Green, 1966; Allmann, Bachmann & Green, 1966; Green, Bachmann, Allmann & Perdue, 1966; Caplan & Greenawalt, 1966; Sottocasa, Kuylenstierna, Ernster & Bergstrand, 1967).

As the interaction of uncoupling reagents with mitochondria can involve binding to sites in addition to those implicated in oxidative phosphorylation (Weinbach & Garbus, 1965), these reagents may interact with and alter the structure of lipoproteins of the outer membrane, as well as interfere with the energy-conservation reactions of the inner mitochondrial membrane. Thus in intact freshly isolated mitochondria, uncoupling reagents may interact with protein sites on the outer mitochondrial membrane, inducing conformational changes that increase the susceptibility to proteolysis, seen as the onset of the rapid decline in extinction.

Gross structural alteration, i.e. the morphological transitions associated with alterations in functional states, and the changes induced by well-known swelling agents, are, however, localized in the inner membrane system (Parsons, 1965; Parsons *et al.* 1966; Hackenbrock, 1966).

Thus we interpret our results as indicating that uncoupling reagents in the minimal concentrations used in this study have a specific affinity for the loci of energy-coupling within the inner membrane system, and that a structural reorganization is associated with the binding of uncoupling reagents at these sites. This is initially observed as an enhanced response to proteolysis. In aged mitochondria with a disrupted outer membrane, the conformational transition results in a delay in proteolysis.

These changes in the rates of proteolysis induced by uncoupling reagents may be related to their action on energy-conservation reactions in mitochondria or may reflect a distinct and diverse property of these reagents. At present there is no way of determining whether structural alterations precede or follow uncoupling of oxidative phos-

phorylation. Although it is conceivable that maintenance of mitochondrial structure is an energetic process, requiring ATP or energized intermediates (Weinbach, 1961b; Lehninger, 1962), it is equally conceivable that some degree of structural order is a prerequisite for coupling phosphorylation to electron transfer. The evidence that we have presented [the interaction of uncoupling reagents with protein sites (Weinbach & Garbus, 1964, 1965), the induction of conformational changes in albumin with these reagents, the increased susceptibility of fresh mitochondria treated with these reagents to proteolytic attack] lends credibility to our view that conformational changes are involved in the uncoupling phenomenon. Resolution of the problem may come from the isolation of the two membrane systems and from determining the response of each to proteinases in the presence and absence of uncoupling reagents.

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