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ENERGY-LINKED CONFORMATIONAL CHANGE OF THE MITOCHONDRIAL MEMBRANE MEASURED WITH THE NH_2-MODIFYING REAGENT, ACROLEIN

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SUMMARY

Application of the NH_2-modifying reagent, acrolein (2-propenal, $CH_2=CHCHO$), to the mitochondrial membrane gave the information that amino groups in the mitochondrial membrane in the energized state are more accessible to acrolein than those in the non-energized state. This finding was supported by the following experimental results. Addition of acrolein to the respiring mitochondria gives rise to rapid H^+ production, which is caused by the reaction of the amino groups in the membrane with acrolein, followed by a slow H^+ consumption, whereas resting mitochondria produce little H^+. The H^+ production is stopped by the addition of NaN_3, antimycin A and 2-thenoyltrifluoroacetone but not by oligomycin. During the course of H^+ production, O_2 consumption and Ca^{2+} uptake remain completely active, indicating that mitochondrial function is unaffected. The subsequent H^+ consumption may be closely related to the destruction of the transmembrane proton gradient formed by mitochondrial respiration.

INTRODUCTION

The conformational change of the mitochondrial membrane induced by the addition of a substrate has been observed by application of various physicochemical techniques: ORD and CD spectrometry[1], spin labeling[2], light scattering[3], fluorochromic analysis[4] and electron microscopic study[5]. There has been little information presented, so far, on the conformational changes of the mitochondrial membrane at the molecular level; there is no answer to the question as to whether changes occur in the mitochondrial protein or lipid, or in both.

During the course of investigations into the higher-order structure of protein, many chemicals which discriminate between the various states of amino acid residues in the protein molecule were explored both in our laboratory[6] and elsewhere[7,8]. Application of these chemicals to the mitochondrial membrane in various states

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, TTFA, 2-thenoyltrifluoroacetone.

may give new information on the mechanism of energization of the mitochondrial membrane.

In the present study, the conformational change of the mitochondrial membrane caused by the transformation from non-energized to energized states was studied by the application of the NH_2-modifying reagent, acrolein (2-propenal, $CH_2=CHCHO$), in order to study the mechanism of the ATP-forming process.

EXPERIMENTALS

Mitochondria were prepared from fresh rat liver by the method described previously[9] and were suspended in 80 mM KCl and 2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.0). Acrolein and antimycin A were purchased from Tokyo Kasei Kogyo Co. and Kyowa Hakko Kogyo Co, respectively. Oligomycin was obtained from Sigma Chemical Co. All other reagents were of analytical grade. Change of pH was measured at 18 °C with a sensitive recording pH meter with expanded scale. Titration of amino acid with alkali was carried out potentiometrically with a Metrom potentiograph E336 with automatic titrator. The reactivity of the mitochondrial membrane or the amino groups of amino acids with acrolein was measured in terms of the amount of induced pH change. Reactivities of functional groups in amino acids with acrolein were measured by a modification of the trinitrobenzene sulfonate method for amino acids[10], the Ellman reaction for sulfhydryl groups[11] and the Sakaguchi reaction for guanidinyl groups[12]. The reactivity of *N*-acetyltyrosine or *N*-acetyltryptophan with acrolein was determined by measuring the absorption spectra of their chromophores. Mitochondrial protein was determined by the biuret method.

RESULTS

Reaction of amino acids with acrolein

Fig. 1 represents the titration curves of glycine with alkali in the presence and the absence of acrolein. The pK_a value, 9.7, of the α-amino group in glycine shifts to a lower value, 8.4, after the modification of α-amino group with acrolein, as shown in curves A and B. The pK_a shift caused by the modification of amino groups with acrolein gives rise to acidification of the medium and is measurable by potentiometric

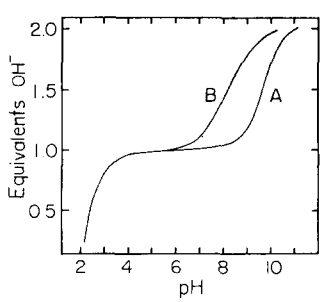


Fig. 1. Titration curve of 50 mM glycine, in the presence and the absence of 0.7 M acrolein, with 2 M NaOH. Curves B and A, glycine with and without acrolein, respectively. Temp. 18 °C.

TABLE I

pK_a SHIFTS, THE AMOUNTS OF H^+ PRODUCTION AND THE REACTIVITIES OF AMINO ACIDS AND THEIR DERIVATIVES WITH ACROLEIN

Amino acid and its derivative	*Functional group*	*pK_a shift**	*H^+ production** (μM)*	*Reactivity***
Glycine	α-Amino	9.7– 8.4	110	+
Serine	α-Amino	9.1– 8.0	216	+
Ethanolamine	Amine	9.5– 7.8	266	+
N-Acetyllysine	ε-Amino	10.1– 8.3	106	+
N-Acetylhistidine	Imidazole	6.8– 6.8	0	–
N-Acetyltyrosine	Phenol	10.1–10.1	0	–
N-Acetyltryptophan	Indole	–	0	–
N-Acetylarginine	Guanidinyl	–	0	–
N-Acetylcysteine	Sulfhydryl	–	0	+

* Reaction mixture of 20 mM each amino acid and 0.5 M acrolein was titrated with 1.0 M NaOH.

** To 10 mM solutions of each amino acid containing 80 mM KCl and 2 mM HEPES buffer (pH 7.0) was added 100 mM acrolein.

analysis. A similar phenomenon was observed in the reaction of amino groups with formaldehyde, and this reaction is widely used in the determination of amino acids by formol titration[13]. Table I shows pK_a shifts, the amounts of H^+ production and reactivities of amino acids and their derivatives with acrolein. The reactivities of the functional groups of amino acids with acrolein are shown in the last column of this table. α-Amino, ε-amino and sulfhydryl groups are reactive, whereas imidazole, phenol, guanidinyl and indole groups are not reactive. Among the functional groups reactive with acrolein, H^+ production is observed in the reaction of α-amino and ε-amino groups with acrolein, but not observed in that of sulfhydryl groups. As is clear from the table, the amounts of H^+ produced from amino groups depend upon the pK_a value of each amino acid modified with acrolein, together with the amount of pK_a shift caused by the modification.

Reaction of mitochondria with acrolein

Addition of acrolein to the respiring mitochondria causes a change of pH in the medium, most likely due to the reaction of amino groups in the mitochondrial membrane with acrolein. The result is shown by Curve A in Fig. 2. The concentration of H^+ in the medium increases with time and tends to approach a constant level 3 min after the addition of 45 mM acrolein to the mitochondrial suspension with 2 mM succinate. Following that, the H^+ concentration decreases gradually to the original level. The upward deflection of the H^+ trace is discussed later. On the other hand, the H^+ production is scarcely observed when 45 mM acrolein is added to the mitochondrial suspension with substrate in the presence of azide. The result is shown by Curve B in the same figure. A similar phenomenon is observed with other inhibitors such as malonate, antimycin A and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The O_2 consumption of the mitochondrial suspension was

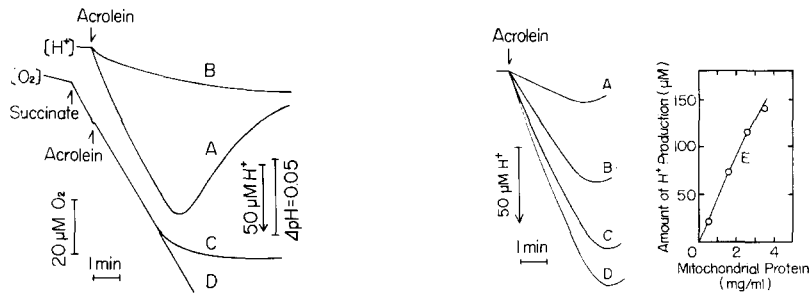


Fig. 2. Change in H^+ and O_2 levels of the mitochondrial suspension with time by the addition of 45 mM acrolein. Curves B and A, change in H^+ level of the mitochondrial suspension (3.5 mg protein/ml) with 2 mM succinate in the presence and the absence of 1 mM azide, respectively. Acrolein was added to the reaction mixture when the succinate-induced H^+ production was completely stopped, which is indicated by the arrow. Curves C and D, change in O_2 level of the mitochondrial suspension (3.5 mg protein/ml) *plus* 2 mM succinate with and without 45 mM acrolein. Acrolein was added at the points indicated by arrows. pH 7.0, temp. 18 °C.

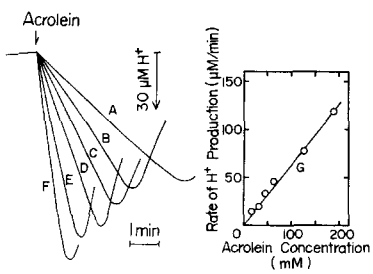
Fig. 3. Relation between the amount of H^+ produced from the mitochondrial membrane by the addition of 50 mM acrolein and the mitochondrial protein concentration. Curves A, B, C and D, mitochondrial membrane containing 0.6, 1.6, 2.6 and 3.5 mg of protein, respectively. Curve E, plot of the amount of H^+ against mitochondrial protein. pH 7.0, temp. 18 °C.

measured under the same experimental conditions as CurveA. As is shown by Curve C, the succinate-induced O_2 consumption is not inhibited by the addition of 45 mM acrolein, but is completely inhibited 3 min after addition of the reagent. The O_2 consumption of the respiring mitochondria without acrolein proceeds linearly with time as shown by Curve D. It is noteworthy that the H^+ production induced by the reaction of amino groups in the mitochondrial membrane in the energized state with acrolein is dramatically greater than in the non-energized state, and that the rate of the succinate-induced O_2 consumption is not affected by the addition of acrolein until the production of H^+ is discontinued.

The amount of H^+ produced by the addition of acrolein was measured as a function of the mitochondrial protein concentration and the result is shown in Fig. 3. As is clear from Curve E, the amount of H^+ produced was proportional to the concentration of mitochondrial protein.

Fig. 4 shows the amount of H^+ produced from the mitochondrial membrane by reacting with acrolein solutions at various concentrations. The rate of H^+ production increases linearly with increasing acrolein concentration, as shown by Curve G in the right-hand panel of the figure.

In order to clarify the phenomenon of the upward deflection of the H^+ trace as seen in Fig. 2, the effect of a proton-conducting uncoupler, such as CCCP, on the change of H^+ caused with acrolein was examined as follows; 1 μM CCCP was added to the reaction mixture during the process of H^+ production or H^+ consumption, and the change of H^+ was recorded. The results are shown in Fig. 5. CCCP was added at the points indicated by the arrows. In each case, an abrupt decrease of H^+ in the medium was observed and the sum of the extents of H^+ decrease induced by CCCP and that of the H^+ decrease induced by acrolein was found to be almost the same, regardless of the time when CCCP was added. Carafoli *et al.*[14]



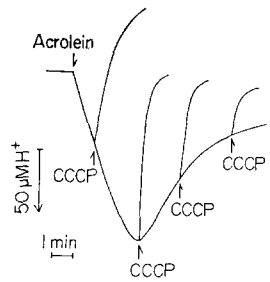


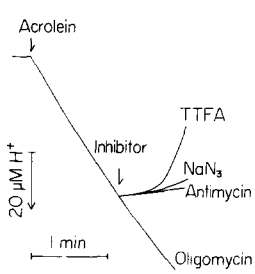
Fig. 4. The rate of H^+ production from the mitochondrial membrane (3 mg protein/ml) induced by acrolein at various concentrations. Curves A, B, C, D, E and F, H^+ production induced by acrolein at 16, 32, 47, 65, 128 and 188 mM, respectively. Curve G, relation between rate of H^+ production and acrolein concentration. Substrate, 2 mM succinate. pH 7.0, temp. 18 °C.

Fig. 5. Effect of CCCP on the acrolein-induced H^+ change in the mitochondrial membrane (3.5 mg protein/ml). CCCP (1 μM) was added at the points indicated by arrows. 2 mM succinate and 50 mM acrolein were used. pH 7.0, temp. 18 °C.

found that the addition of uncouplers such as CCCP, dinitrophenol or *p*-trifluoromethoxycarbonylcyanide phenylhydrazone to respiring mitochondria gives an increase in the uptake of H^+.

Fig. 6 shows the effect of various inhibitors on the H^+ production. The addition of respiratory inhibitors such as antimycin A, NaN_3 or 2-thenoyltrifluoroacetone (TTFA) inhibits the H^+ production, while the addition of oligomycin, an energy transfer inhibitor, does not affect the H^+ production at all. The result of this experiment suggests that the acrolein-induced H^+ production is a consequence of the energized state of mitochondria.

It is known that the addition of Ca^{2+} to the mitochondrial suspension with substrate causes an accumulation of Ca^{2+} in mitochondria and an ejection of H^+



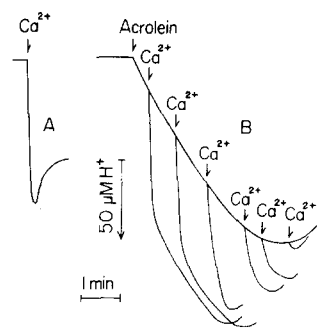


Fig. 6. Effect of various inhibitors on the acrolein-induced H^+ production from the mitochondrial membrane (3.5 mg protein/ml). Concentrations of the inhibitors are as follows; 1 μM TTFA, 1 mM NaN_3, 0.5 μM antimycin A and 2 μg/ml oligomycin. 2 mM succinate and 50 mM acrolein were used. The addition of each inhibitor is indicated by the arrows. pH 7.0, temp. 18 °C.

Fig. 7. Effects of Ca^{2+} on the process of the acrolein-induced H^+ production from the mitochondrial membrane (Curve B). 3.5 mg protein/ml, 2 mM succinate, 50 mM acrolein and 50 μM Ca^{2+} were used. pH 7.0, temp. 18 °C. Curve A, H^+ ejection into the medium when adding Ca^{2+} to the mitochondrial suspension with 2 mM succinate.

into the medium[15]. In the process of the acrolein-induced H^+ production, 50 μM Ca^{2+} was added to the reaction mixture and the change of H^+ was measured with time. As is clear from Curve B, each time Ca^{2+} were added to the reaction mixture, rapid H^+ ejection was observed immediately after the addition of Ca^{2+}, followed by slow H^+ ejection. On the other hand, the addition of Ca^{2+} to the reaction mixture in a state without H^+ production caused no H^+ ejection at all. These results imply that the mitochondrial function becomes completely disrupted when the mitochondrial membrane reaches the state where H^+ production does not take place, but the function is hardly disrupted by acrolein during the course of H^+ production.

DISCUSSION

In the present study, acrolein has been found to be a useful probe for investigating the conformation of the mitochondrial membrane; it has been shown that the conformation of the mitochondrial membrane in the energized state is quite different from that in non-energized state, as measured by the reactivity of amino groups present in the mitochondrial membrane with acrolein.

The application of chemicals with aldehyde groups, such as formaldehyde and glutaraldehyde, in studies on the conformation of protein and mitochondria was carried out by French and Edsall[13] and Fortes[16].

These chemicals react with amino groups in organic substances, and the reaction mechanism has been described elsewhere[13]. It was reported by Smith and Packer[17] that, in mitochondria, formaldehyde and glutaraldehyde were oxidized by the respiratory system but acrolein is not, probably due to its double bond. On the bases of the information mentioned above, acrolein is employed as NH_2-modifying reagent in order to investigate the structure and the function of mitochondria. Acrolein reacts with amino and sulfhydryl groups at pH 7.0, but H^+ production occurs only by the reaction of amino groups and not of sulfhydryl groups. Therefore, the H^+ production caused by the addition of acrolein to the mitochondrial suspension is most likely due to the amino groups present in mitochondria.

The change of pH adding acrolein to the mitochondrial suspension with succinate is caused by an abrupt production of H^+ for 3 min followed by a slow consumption of H^+. The time course of H^+ production is similar to that of O_2 consumption, indicating that the reaction of acrolein with amino groups in mitochondria does not cause the destruction of the mitochondrial function during the first 3 min after adding acrolein. This is also supported by the experimental data shown in Fig. 7 with regard to the H^+ ejection caused by the addition of Ca^{2+} in the course of the acrolein-induced H^+ production. The upward deflection of the H^+ trace occurring 3 min after the addition of acrolein can be explained as follows from the experiment concerning the H^+ consumption induced by adding CCCP in the course of the acrolein-induced H^+ production (Fig. 5); the electrochemical gradient across the membrane formed by the respiring mitochondria is discharged by the modification of the mitochondrial membrane with acrolein.

The most important finding in the present study is that there exists a great difference between the amount of H^+ produced from mitochondria with azide and the amount produced from the respiring mitochondria without azide (Curves A and B in Fig. 2). This implies that the mitochondrial membrane in the energized

state is more accessible to the reagent than when in non-energized state. It also indicates that a remarkable conformational change of the mitochondrial membrane occurs by the transformation from non-energized to energized state. A similar phenomenon was also demonstrated by measuring the spectral change of bixin (carotenoid carboxylic acid), bound to the mitochondrial membrane, caused by the addition of substrate, according to reports by Inada and his co-workers[9,18]. There still remains the possibility that the reaction of acrolein with the mitochondrial membrane in the non-energized state proceeds to the same degree as occurs with the mitochondrial membrane in energized state, but the H^+ production from the membrane does not take place due to the reaction of acrolein with amino groups present in a hydrophobic region of the mitochondrial membrane.

As mentioned in the Introduction, the reaction of amino groups with acrolein may occur in the mitochondrial protein or in the mitochondrial lipids such as phosphatidylethanolamine and phosphatidylserine. Experiments are in progress to clarify this point.

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