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Kinetics of the Resolution of Complex I (Reduced Diphosphopyridine Nucleotide–Coenzyme Q Reductase) of the Mitochondrial Electron Transport System by Chaotropic Agents*

K. A. Davis and Y. Hatefi

ABSTRACT: Chaotropic agents (NaSCN, $NaClO_4$, guanidine-HCl, and urea) resolve the enzymic assembly of complex I (reduced diphosphopyridine nucleotide–coenzyme Q reductase) resulting in the solubilization of the reduced diphosphopyridine nucleotide dehydrogenase and the iron–sulfur protein of the complex. They also cause the release of these components from the binary I–III complex and electron transport particle. The resolution of complex I is first order with respect to the appearance of soluble reduced diphosphopyridine nucleotide dehydrogenase and highly temperature dependent at low concentrations of chaotropic agents. As in the case of microsomes and other complexes of the respiratory chain, the aerobic resolution of complex I is accompanied by destruction of the iron–sulfur proteins and considerable lipid oxidation. However, under anaerobic conditions the resolution of complex I by chaotropic agents proceeds in the absence of any detectable lipid oxidation or iron–sulfur protein destruction. Reduced diphosphopyridine nucleotide, but not diphosphopyridine nucleotide or reduced triphosphopyridine nucleotide, impedes the chaotropic-induced resolution of complex I, suggesting that the reduced complex has a more stable conformation.

Recent studies in this laboratory have shown that anions such as SCN^-, ClO_4^-, and I^- (chaotropic agents) disrupt membrane structure and increase the water solubility of particulate proteins and nonelectrolytes (Hatefi and Hanstein, 1969). Physicochemical considerations have permitted the interpretation that chaotropic agents change the structure and the lipophilicity of water such that membrane hydrophobic bonds, which are mainly responsible for the stability of such structures in aqueous media, are weakened and the thermodynamic barrier for entry of nonelectrolytes and the apolar groups of proteins into the aqueous phase is lowered. These studies have further demonstrated that guanidine-HCl and urea[1] also behave like chaotropic ions in solubilization of particulate proteins and nonelectrolytes. In addition it has been shown that as a consequence of membrane resolution, chaotropic agents induce a very rapid lipid oxidation in ETP,[2] the purified complexes of the respiratory chain, and microsomes.

The resolution of complex I by chaotropic agents has yielded (a) a DPNH dehydrogenase, containing 1 mole of flavin, 4 g-atoms of iron, and 4 moles of labile sulfide per 70,000 g of protein; (b) an iron–sulfur protein; and (c) a protein fraction with solubility properties similar to the mitochondrial "structural" proteins (Hatefi and Stempel, 1967, 1969). In this process, the catalytic and inhibitor-response properties of DPNH dehydrogenase undergo vast changes as the particle-bound enzyme is rendered soluble. These changes include a tenfold increase in K_m^{DPNH}, the emergence of very high reductase activities with respect to quinones (menadione and DCIP) and ferric complexes (ferricyanide and cytochrome *c*) as electron acceptors and the appearance of an active thiol (Hatefi and Stempel, 1969).

The present study describes the kinetics of the resolution of

* From the Department of Biochemistry of Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received March 31, 1969.* This work was supported by U. S. Public Health Service Grant RO1-AM08126.

[1] Hatefi and Hanstein (1969) have defined chaotropic agents as "those inorganic anions which favor the transfer of apolar groups to water." However in this paper guanidine-HCl and urea will also be referred to as chaotropic agents.

[2] Abbreviations used are: complex I, particulate, rotenone-, and piericidin A sensitive DPNH–coenzyme Q reductase; the binary I–III complex, particulate DPNH–cytochrome *c* reductase; ETP, electron transport particle; DCIP, 2,6-dichlorophenolindophenol; MDA, malondialdehyde.

complex I as a function of (a) nature and concentration of various chaotropic agents, (b) temperature of the reaction medium, and (c) presence or absence of substrate and oxygen. Furthermore, these studies demonstrate that, by contrast to other techniques heretofore used (various combinations of organic solvents, detergents, sonic, and shearing forces, phospholipases, proteolytic enzymes, and extremes of pH), the chaotropic-induced resolution of particulate enzyme systems can be performed with a considerable degree of control, and provide a model for the resolution of enzyme complexes and membrane assemblies by the novel use of chaotropic agents.

Materials and Methods

1. Assays. The assay conditions for DPNH dehydrogenase were the same as reported previously (Hatefi and Stempel, 1967), except that menadione reductase activity was measured in the presence of 0.2 mM menadione with Tris-HCl as buffer and DCIP reductase activity was measured in the presence of 0.065 mM DCIP.

Iron was estimated essentially according to the method of Doeg and Ziegler (1962). Acid-labile sulfide was estimated by the procedure of Fogo and Popowsky (1949) as outlined before (Hatefi and Stempel, 1969). Malondialdehyde was determined essentially as described by Heath and Packer (1968) and protein was estimated by the biuret method of Gornall *et al.* (1949). Absorption spectra and enzyme activities were recorded by a Beckman DK-2A spectrophotometer fitted with a time-drive attachment. Specific activities are expressed as micromoles of DPNH oxidized per minute times milligrams of protein at 38°. Complex I (Hatefi *et al.*, 1962) and the binary I–III complex (Hatefi *et al.*, 1961) were prepared as previously reported. ETP was prepared from beef heart mitochondria by sonication and differential centrifugation. DPNH, DPN, and TPNH were obtained from Pabst Laboratories. All other chemicals were reagent grade.

2. Resolution of Complex I. The resolution of complex I was performed by one of the following methods.

A. AEROBIC INCUBATION AND DILUTION. Preparations of complex I, suspended in a solution containing 50 mM Tris-HCl (pH 8.0), 0.66 M sucrose, and 1 mM histidine (TSH), were preincubated at the desired temperature for 3 min. At time zero a concentrated TSH solution of the chaotropic agent (*e.g.*, 4 M $NaClO_4$ in TSH) was added to the desired molarity. At this point protein concentration in this and in methods B and C was 10 mg/ml. At various time intervals, aliquots of the reaction mixture were taken and diluted 20-fold in ice-cold TSH. This treatment arrests the resolution process immediately. The diluted samples were then assayed for DPNH dehydrogenase activity. The activities did not change even after 2–3 hr at 0°.

B. AEROBIC INCUBATION AND CENTRIFUGATION. Suspensions of complex I in TSH and chaotropic agents were prepared exactly as in method A except that incubations were performed in 2-ml ultracentrifuge tubes for various lengths of time. Those to be incubated longest were started first so that all tubes could be removed from the water bath at the same time. The tubes were then chilled in an ice bath for 3 min and centrifuged at 105,000*g* for 20 min. The resulting supernatants were decanted from a firm pellet and assayed. For direct comparison with method A specific activities were calculated on the basis of the original protein concentration (*i.e.*, 10 mg/ml) before centrifugation.

C. ANAEROBIC INCUBATION. Complex I suspensions in a total volume of 2 ml were placed in Thunberg tubes with the appropriate amount of chaotropic agent in the side arm. The tubes were sealed and carefully evacuated and filled with argon six to seven times. After a 5-min preincubation at the desired temperature, the chaotropic agent was added from the side arm and the incubation was continued. After various time intervals the tubes were placed in an ice bath and where indicated 1-ml aliquots were assayed immediately for MDA.[3] The remainder of the samples was then centrifuged at 105,000*g* for 20 min and the resulting supernatants were assayed for DPNH dehydrogenase activity. Once again, for purposes of comparison, specific activities were calculated on the basis of the original protein concentration. Aerobic controls were run in an identical manner without evacuation and gassing with argon. In several experiments an attempt was made to remove the last possible traces of oxygen by preincubating the suspension in the presence of ascorbate and catalytic amounts of cytochrome *c* and purified cytochrome oxidase prior to addition of the chaotropic agent. The results were the same as those which were only evacuated and gassed with argon.

Results

1. Resolution of Complex I and Parent Particles by Chaotropic Agents. Previous studies have shown that chaotropic agents disrupt the membrane structure of the mitochondrial electron transport system and cause considerable protein solubilization (Hatefi and Hanstein, 1969). For example, about 70% of the protein of ETP can be solubilized by 2 M $NaClO_4$ and 30% of the protein of complex I can be solubilized by as little as 0.5 M $NaClO_4$. The soluble protein fraction obtained from complex I is composed mainly of DPNH dehydrogenase and an iron–sulfur protein. When bound to the particulate assembly of complex I, the dehydrogenase exhibits little or no reductase activity with respect to menadione (2-methylnapthoquinone), DCIP, and cytochrome *c*. Moreover its ferricyanide reductase activity is (a) insensitive to mercurials and (b) has a K_m^{DPNH} of 7 μM and a $K_m^{Fe(CN)_6^{3-}}$ of 4000 μM. In the soluble form the dehydrogenase acquires very high reductase activities with respect to the above electron acceptors, its ferricyanide reductase activity (as well as its menadione, DCIP, and cytochrome *c* reductase activities) becomes sensitive to mercurials and is marked by a K_m^{DPNH} of 65 μM and a $K_m^{Fe(CN)_6^{3-}}$ of 1650 μM (Hatefi and Stempel, 1969). Thus the conversion of particle-bound DPNH dehydrogenase into the soluble form can be monitored by the above changes.

Figure 1 shows the appearance of menadione, DCIP, cytochrome *c*, and mercurial-sensitive ferricyanide reductase activities as complex I is incubated at 30° in the presence of 0.45 M $NaClO_4$. It is seen that (a) the efficiency of electron transfer to these acceptors is the same as shown previously for purified preparations of DPNH dehydrogenase (Hatefi and Stem-

[3] In cases where malondialdehyde assays were to be performed, complex I was suspended in 50 mM Tris-HCl (pH 8.0) as sucrose interferes with the MDA assay.

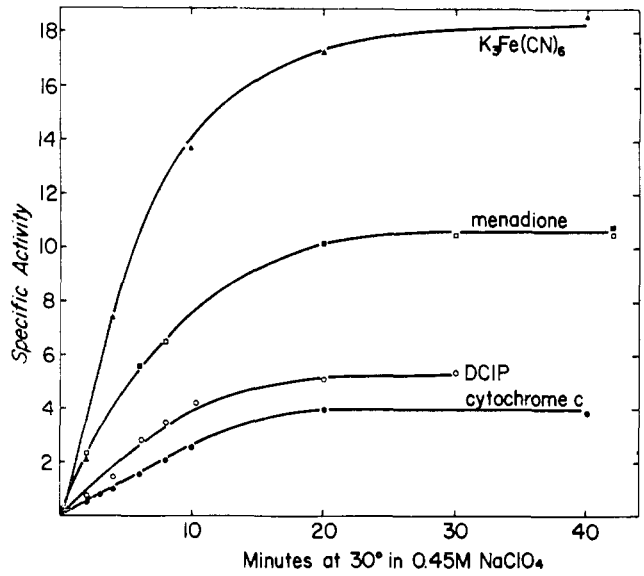

FIGURE 1: The appearance of ferricyanide, menadione (tested by both methods 2A and 2B), DCIP, and cytochrome *c* reductase activities as a function of incubation of complex I in 0.45 M $NaClO_4$ at 30°. All experiments were conducted as described in section 2A of Methods, except the appearance of ferricyanide reductase activity (▲—▲) and menadione reductase activity (■—■), which were monitored as described in section 2B of Methods. The menadione and DCIP reductase activities (1.5 and 0.86, respectively) of the unresolved complex I were subtracted from the values given in the figure. Cytochrome *c* reduction was assayed in the presence of 2 μM antimycin A.

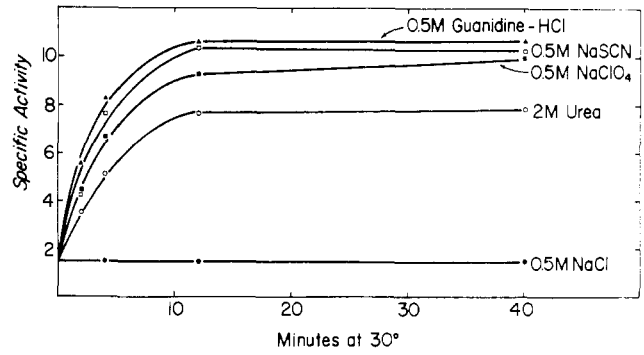

FIGURE 2: The appearance of menadione reductase activity as a function of incubation of complex I at 30° with various chaotropic agents.

pel, 1967, 1969), (b) the relative emergence of these activities is essentially the same at various stages of incubation, and (c) all the activities so produced reach a maximum after about 15-min incubation.[4] Figure 1 also shows that the emergence of the above activities parallels the solubilization of DPNH dehydrogenase. This point is demonstrated in the measurement of menadione reductase activity before (open squares) and after (filled squares) removal of the insoluble material by centrifugation (see resolution procedures A and B in Methods).

Figure 2 shows the resolution of complex I and the appearance of soluble DPNH dehydrogenase as induced by guanidine-HCl, NaSCN, $NaClO_4$, or urea. It is seen that guanidine-HCl and NaSCN are slightly more effective in the resolution of complex I than $NaClO_4$, and that considerably higher concentrations of urea (2 M as compared with 0.5 M of the aforementioned reagents) are required for the resolution of this complex. For comparison, the effect of 0.5 M NaCl is also shown. It is seen that after 40-min incubation at 30°, NaCl is completely ineffective. The order of the chaotropic potency of guanidine-HCl, NaSCN, $NaClO_4$, and urea is essentially the same as has been shown by Hatefi and Hanstein (1969) for a variety of phenomena, including depolymerization of biopolymers and solubilization of particulate proteins and nonelectrolytes. Among the above chaotropic agents, $NaClO_4$ is the most stable, the most inert under the conditions used and most easily obtained in pure form. At the concentrations used, it has the least deleterious effect on the enzymes under consideration, and has the advantage that it is readily removable not only by dialysis but also as an insoluble potassium salt. Therefore, in the experiments reported in this communication, $NaClO_4$ was used as the chaotropic agent of choice.

As pointed out above, the DPNH dehydrogenase and the iron–sulfur protein of complex I are solubilized by chaotropic agents. Figure 3A shows the appearance of nonheme iron and acid-labile sulfide (from both DPNH dehydrogenase and iron–sulfur protein) in the soluble fraction of complex I at different times during incubation of the complex with $NaClO_4$. It is seen that the release of these components from the particles parallels the appearance of dehydrogenase activity in the supernatant fraction. In addition, Figure 3A shows data obtained from the change in absorbancy of the soluble fraction of complex I at 450 nm (ΔA_{450}) after reduction with $Na_2S_2O_4$. This change is another measure of the release of DPNH dehydrogenase and iron–sulfur protein into the soluble fraction as both of these components exhibit a maximum absorbancy change at about 450 nm upon dithionite reduction (see Figure 3B for the spectra of urea-induced solubilization of these components at 0-, 4-, 8- and 12-min incubation at 30°; (Hatefi and Stempel, 1967). Since the solubilization of the iron–sulfur protein also occurs during the incubation period shown in Figure 3A, these data suggest that, as far as can be determined, both DPNH dehydrogenase and the iron–sulfur protein of complex I are concomitantly released by chaotropic agents. If this were not the case, one might have expected an unparallelism at some stage of the resolution process between the emergence of dehydrogenase activity and the appearance of iron, acid-labile sulfide, and the 450-nm reducible material (ΔA_{450}) in the supernatant fraction.

That the release of DPNH dehydrogenase by chaotropic agents is not peculiar to complex I is demonstrated by the experiments of Figure 4. It is seen that essentially the same type of kinetics of the appearance of dehydrogenase activity is obtained when the binary I–III complex (the immediate parent particle of complex I) or ETP is used as the source of the particle-bound enzyme. The differences in the final activities shown in Figure 4 are only a reflection of the dehydrogenase concentration of each particle. For example, ETP preparations con-

[4] As pointed out in Materials and Methods, the specific activities shown in the figures are on the basis of the total protein of complex I. The purified DPNH dehydrogenase catalyzes the reduction of ferricyanide, menadione, DCIP, and cytochrome *c* at rates corresponding to 215, 170, 100, and 43 μmoles of DPNH oxidized per min × mg of protein at 38°, respectively (Hatefi and Stempel, 1967, 1969).

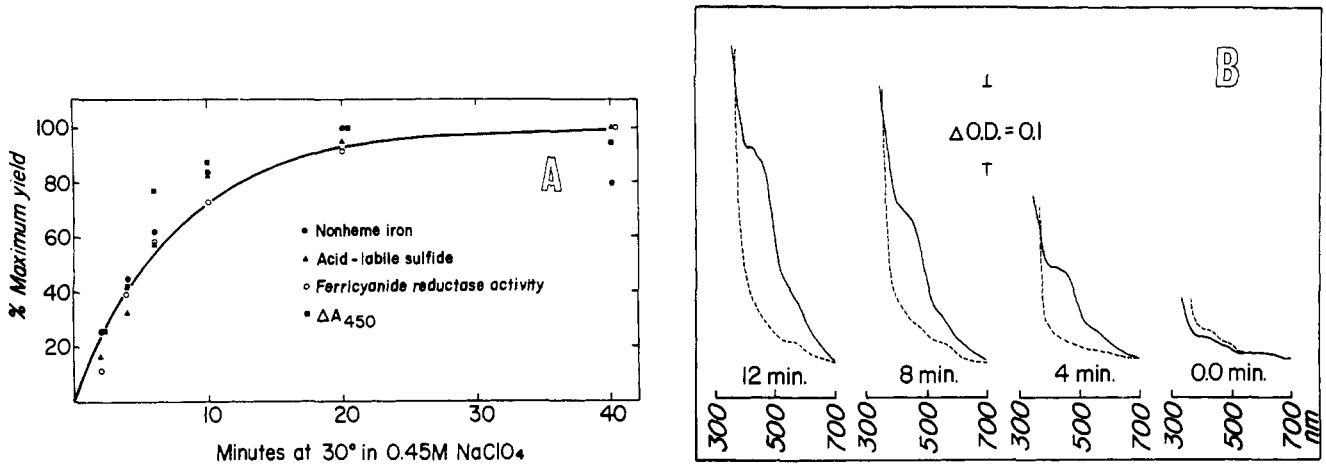

FIGURE 3: (A) The release of DPNH dehydrogenase activity and the components of DPNH dehydrogenase and iron-sulfur protein from complex I. All assays were performed on the supernatant fraction obtained as described in section 2B of Methods. (B) Oxidized and dithionite-reduced spectra of the soluble fraction of complex I released by incubation with 2.2 M urea at 30°. The dithionite-reduced spectra are dashed.

tain only 0.23 nmole (Green and Wharton, 1963) of FMN (the coenzyme of DPNH dehydrogenase) per mg of protein while complex I contains 1.4 nmoles of FMN/mg of protein, and if the final activity of 1.7 derived from ETP is multiplied by 1.4/0.23 the result is 10.3, which is quite comparable with the activity of 10 obtained from complex I. It should also be noted that, unlike complex I and the binary I–III complex, the ETP preparations used in the experiments of Figure 4 were never treated with detergents, thus indicating that the presence of detergent is not necessary for the process of chaotropic-induced resolution.

II. Kinetics of the Resolution of Complex I. As seen in Figure 5, the release of soluble DPNH dehydrogenase from complex I is first order during incubation at 30°, and increasing concentrations of $NaClO_4$ have a substantial effect on the rate of resolution of complex I. The latter phenomenon is better demonstrated in Figure 6 where the rate constants calculated from the slopes of first-order traces such as those shown in Figure 5 are plotted against the concentration of $NaClO_4$. It is seen that at 30° very little resolution occurs as the concentration of $NaClO_4$ is increased from 0.15 to 0.4 M, but that beyond 0.4 M there is a dramatic increase in the rate of resolution. Similar results are obtained at 20°, except that the break in the rate of resolution occurs at a concentration of about 0.76 M $NaClO_4$. In addition to their intrinsic value (see below) these results clearly demonstrate the degree of control that can be applied to the resolution of an enzyme complex and the accuracy with which the kinetics of a resolution process can be studied.

III. Factors Influencing the Resolution of Complex I. 1. TEMPERATURE AND pH. At moderate concentrations of chaotropic agents, the resolution of complex I is dramatically af-

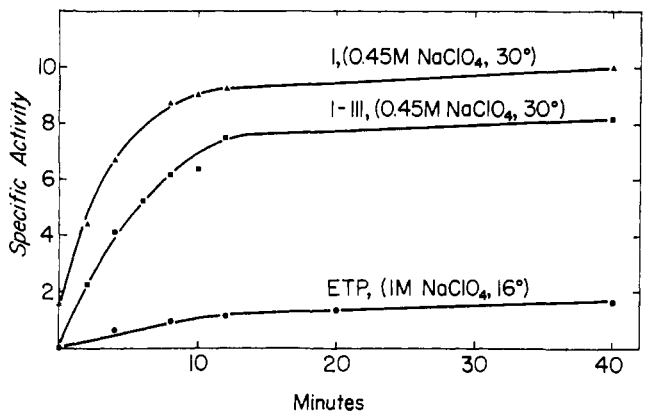

FIGURE 4: The release of DPNH dehydrogenase from various submitochondrial particles by $NaClO_4$. The incubations were conducted as described in section 2A of Methods, and soluble DPNH dehydrogenase activity was assayed in the presence of menadione as electron acceptor.

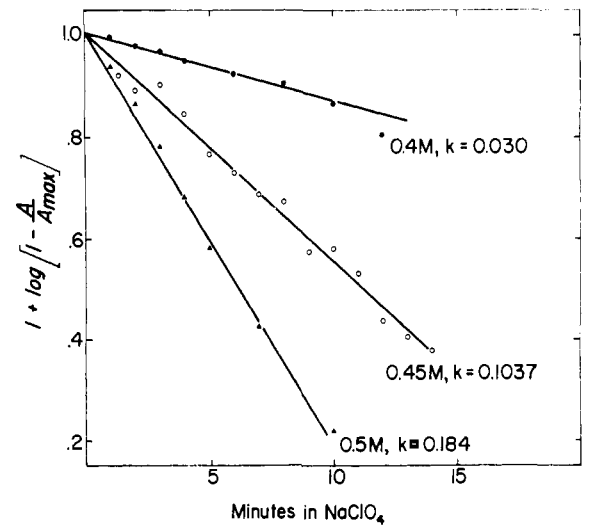

FIGURE 5: First-order plot of the kinetics of complex I resolution at various concentrations of $NaClO_4$. The experiments were conducted as described in Figure 4. Data were calculated from the first-order equation $1 + \log(1 - (A/A_{max})) = (-kt/2.303) + 1$, where A is specific activity at time t, A_{max} is maximum dehydrogenase activity releasable from complex I, and k is the first-order reaction rate constant in $min^{-1} \times$ mg of $protein^{-1}$. For the convenience of plotting, a factor of unity has been added to both sides of the first-order equation as seen above. The rate constants shown were calculated from $k = -2.303 \times$ slope.

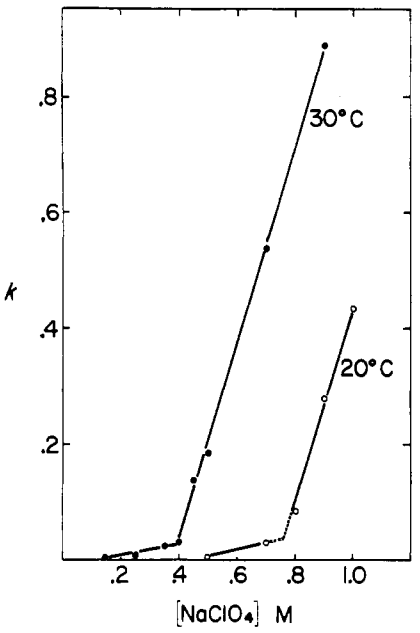

FIGURE 6: The kinetics of the resolution of complex I at 20 and 30° as a function of $NaClO_4$ concentration. The experimental conditions were the same as in Figure 4, and the first-order rate constants, k, were calculated as described in Figure 5.

fected by the temperature of the reaction mixture. Thus, as seen in Figure 7, the resolution at 30° in the presence of 0.47 M $NaClO_4$ can be almost completely stopped if the reaction mixture is cooled to about 15° or less. This temperature effect is also demonstrated in Figure 6, where it is shown that at 20° very little resolution occurs in the presence of $NaClO_4$ concentrations between 0.5 and 0.75 M, whereas at 30° and the same range of $NaClO_4$ concentration, the rate of resolution is 20–40 times as fast.

Using the Arrhenius equation and the data of Figure 6, activation energy changes were calculated for the temperature change from 20 to 30°. Such calculations indicate a decrease of about 40,000 cal/mole as the concentration of $NaClO_4$ is increased from 0.6 to 1.0 M. This tremendous drop in activation energy associated with an increase in the concentration of $NaClO_4$ is consistent with the interpretation of Hatefi and Hanstein (1969) regarding the mechanism of action of chaotropic ions and their unique ability to increase the water solubility of particulate proteins and nonelectrolytes. The effect of chaotropic agents in lowering the activation energy of the resolution of complex I is in essence similar to the observation of Hamaguchi and Geiduschek (1962) who found that ions such as SCN^- and ClO_4^- lower the thermal denaturation temperature of DNA at neutral pH by as much as 50°.[5]

Since chaotropic compounds such as NaSCN, $NaClO_4$, guanidine-HCl, and urea are all neutral, it might be expected that moderate pH changes would have very little effect on the activity of these compounds. This is true at the pH range 6–9; however, below pH 6 the resolution is more rapid. This increase in the rate of resolution might be associated with the

[5] Both the magnitude of the activation energy changes and the shape of traces in Figure 6 are also somewhat reminiscent of protein denaturation data, and phenomenologically the resolution of complex I is not too unlike the unfolding of a protein molecule.

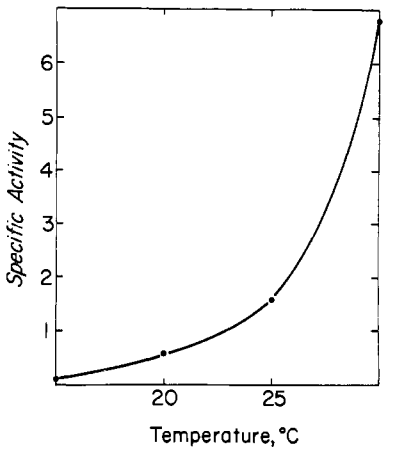

FIGURE 7: The kinetics of the resolution of complex I as a function of the temperature of the incubation medium. The release of menadione reductase activity was measured 2.5 min after incubation of complex I with 0.47 M $NaClO_4$ at the temperatures indicated.

instability of complex I in an acid medium. In fact, the original extraction of the mitochondrial DPNH dehydrogenase involved the use of 10% ethanol at pH 4.8 (Mahler *et al.*, 1952; de Bernard, 1957).

2. THE EFFECT OF OXYGEN. As shown elsewhere (Y. Hatefi and W. G. Hanstein, 1969, in preparation), the disruption of membrane structure by chaotropic agents is accompanied by a very rapid oxidation of the lipids, which can be monitored either by measuring oxygen consumption or by the formation of malondialdehyde, a product of the oxidation of fatty acids containing 1,4-dienes. Concomitant with the oxidation of lipids the iron–sulfur proteins of mitochondria are also destroyed leading to a loss of acid-labile sulfide. The studies of membrane lipid oxidation induced by aging or ionizing radiation (reviewed by Packer *et al.*, 1967) have not explained whether membrane breakdown precedes or follows the oxidation of lipids. This distinction, which has significant implications in the understanding of membrane senescence, is easily possible through the use of chaotropic agents. Thus, our studies show that in complex I the disruption of mem-

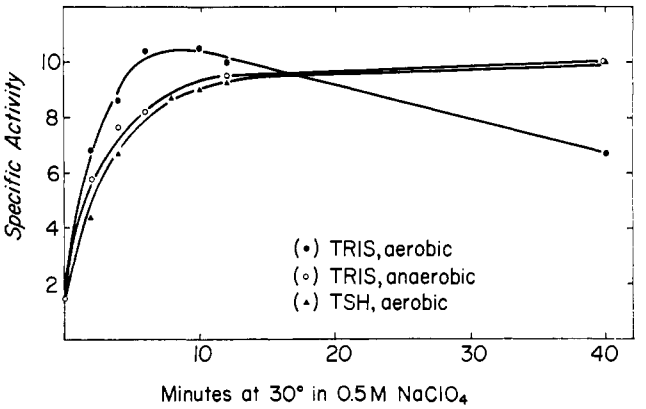

FIGURE 8: The effect of anaerobiosis and high sucrose concentation on the resolution of complex I and the stability of released products. Incubation conditions were as described in sections 2A and 2C of Methods, and the release of DPNH dehydrogenase was monitored by the appearance of menadione reductase activity.

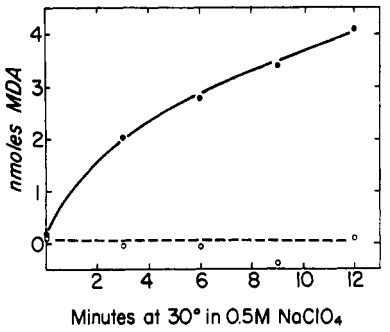

FIGURE 9: The appearance of malondialdehyde as a function of incubation of complex I with $NaClO_4$. For MDA analysis samples were taken at the intervals shown from the incubation mixtures (●) and (○) of Figure 8.

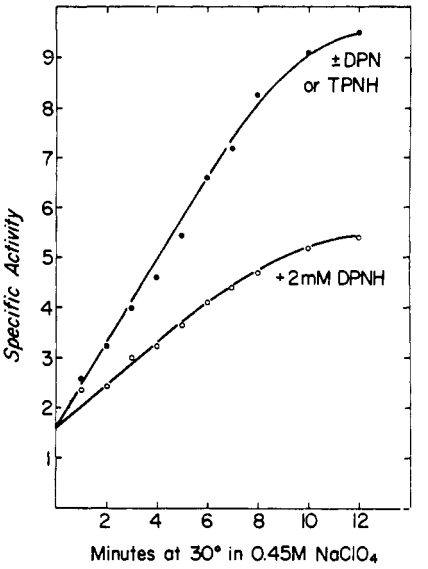

FIGURE 10: The inhibition of $NaClO_4$ induced resolution of complex I by DPNH. Experimental conditions were the same as in Figure 4.

brane structure is independent of lipid oxidation and can proceed under anaerobic conditions. As seen in Figure 8, the resolution of complex I and the appearance of DPNH dehydrogenase activity can be induced by 0.5 M $NaClO_4$ in the presence or absence of air. However, under anaerobic conditions no malondialdehyde is formed (Figure 9), and after 12-min incubation no loss of acid-labile sulfide can be detected. Figure 8 also shows a loss of dehydrogenase activity as a result of prolonged incubation at 30° under aerobic conditions. This loss of activity is impeded in the presence of high sucrose concentration (0.66 M) as is membrane lipid oxidation induced by chaotropic agents.

3. THE EFFECT OF SUBSTRATE. Recent electron microscopic studies of mitochondria (Hackenbrock, 1966; Harris *et al.*, 1968) have shown marked changes in the conformation of cristae, which are apparently associated with various metabolic states of the mitochondria. Such changes have not been reported, however, for submitochondrial particles. As seen in Figure 10, 2 mM DPNH inhibits the resolution of complex I considerably, whereas DPN or TPNH have no effect. Since the action of chaotropic agents appears to be primarily on the surrounding water rather than on the membranes themselves (Hatefi and Hanstein, 1969), the above results suggest that the reduced state of complex I has a more stable conformation than the oxidized form. Consequently the resolution of the reduced system by chaotropic agents is less efficient. Studies of chaotropic-induced lipid oxidation in ETP and the complexes of the electron transport system also support this conclusion (Hatefi and Hanstein, 1969, in preparation). Furthermore, it has been shown in this laboratory that the extraction of succinate dehydrogenase from succinate–coenzyme Q reductase is inhibited in the presence of substrate (Baginsky and Hatefi, 1969).

Discussion

It has been shown that chaotropic agents resolve the enzymic assembly of complex I and solubilize the DPNH dehydrogenase and the iron–sulfur protein of this complex.[6] The chaotropic-induced resolution of complex I is first order with respect to the appearance of DPNH dehydrogenase, and can be made to proceed at a faster or a slower rate by adjusting the concentration of the chaotropic agent used, or by changing the temperature of the reaction medium. The resolution process can be interrupted by cooling of the reaction mixture and separation of the resolved products by differential centrifugation. These results clearly show that the potency of chaotropic agents and the precision with which they can be used for the resolution of enzyme complexes and membrane assemblies are unmatched by any technique used previously. As discussed elsewhere (Hatefi and Hanstein, 1969) the action of chaotropic agents appears to be primarily on the structure and lipophilicity of the surrounding water. Therefore, it might be expected that chaotropic agents should have wide applicability for the resolution of various enzyme systems.[7] In this respect the results shown above serve as a model for the use of this novel technique.

The degree of control afforded by the use of chaotropic agents has also permitted the following observations: (a) that DPNH specifically impedes the resolution of complex I, which in view of the mechanism of action of chaotropic agents suggests a DPNH-induced change in the complex in the direction of a more stable structure. Such structural stabilization occurring at the level of the individual complexes of the

[6] The release and isolation of iron–sulfur protein from complex I have been described elsewhere (Hatefi and Stempel, 1967). In this paper the concomitant release of iron–sulfur protein and DPNH dehydrogenase is only demonstrated as shown in Figure 3 by monitoring the appearance of nonheme iron and acid-labile sulfide in the soluble fraction of complex I.

[7] Data compiled by Kauzmann (1959) show that antigen–antibody complex information, the conversion of insulin monomer into dimer and trimer, and the interaction of serum albumin with detergents (fatty acyl sulfate) and dyes (methyl orange, azosulfathiazole, etc.) are associated with unitary entropy changes of about +15 to +30 eu, and that at room temperature $T\Delta S_u > \Delta H$. These data agree with the interpretation of Hatefi and Hanstein (1969) that chaotropic anions lower the entropic barrier for entry of apolar groups (*e.g.*, of proteins) into water and suggest by analogy that the stability of complex I in aqueous media might be due to a large negative entropy of resolution.

electron transport system might be associated with subtle conformation changes as gross conformation changes effected by substrates have been observed with the electron microscope at the level of whole mitochondria (Hackenbrock, 1966; Harris *et al.*, 1968). (b) That unless care is taken to eliminate oxygen, a considerable degree of lipid oxidation and iron–sulfur protein destruction can occur during membrane resolution. In the methods used previously for fragmentation of membranes, these hazards were neither detected nor eliminated, and a total reconstitution of the mitochondrial electron transport and oxidative phosphorylation system might well depend upon such precautions.

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