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Respiration-Dependent Proton Movements in Rat Liver Mitochondria*

Alan M. Snoswell

ABSTRACT: Rat liver mitochondria were found to extrude protons into the medium at a constant rate when incubated in various media. Proton extrusion was measured with a sensitive pH electrode system. The addition of adenosine diphosphate (ADP) to mitochondria respiring in a medium containing magnesium and phosphate caused an immediate halt in the liberation of protons and, as expected, H^+ concentration decreased during the phosphorylation of the ADP. In all media the extrusion of protons was dependent on respiration. It did not occur in the presence of antimycin or cyanide or in the absence of oxygen. It was not noticeably affected by the presence of other ions such as Mg^{2+}, $H_2PO_4^-$, Na^+, or K^+. The rate of proton extrusion varied between 10 and 100% of the rate of electron transport under varying conditions. Uncoupling agents increased the rates of both respiration and proton extrusion but substantially decreased the ratio of the rate of proton extrusion to the rate of electron transport. In the presence of cyanide and ferricyanide the rate of proton extrusion was approximately equal to the rate of electron transport. These results suggest that proton extrusion is a normal mitochondrial process and that the protons probably arise as direct products of respiration.

During the past few years a considerable number of reports have been published dealing with the translocation of various ions in mitochondrial preparations. While most of these reports have dealt with the respiration-dependent movement of Ca^{2+}, $H_2PO_4^-$, Mg^{2+}, Mn^{2+}, Na^+, and K^+, in a number of cases it was shown that there was an extrusion of protons from the mitochondria as other ions were taken up. It has been proposed that protons may be released from the mitochondria due to the deposition of insoluble salts within the mitochondria (*e.g.*, Brierley *et al.*, 1962) or by an exchange reaction with other cations (*e.g.*, Chappell and Greville, 1963; Chance, 1965). Such an exchange reaction for K^+ or Na^+ apparently requires the presence of an uncoupling agent (Moore and Pressman, 1964; Chappell and Crofts, 1965). These movements of protons are generally considered to be secondary reactions resulting from the movement of other cations. However, Chappell and Crofts (1965) suggested that a respiration-dependent H^+ pump mechanism might provide the underlying mechanism for mitochondrial ion movements. Mitchell (1963) has suggested that the separation of protons and OH^- ions on opposite sides of the mitochondrial membrane during respiration provides the driving potential for the synthesis of ATP.[1]

Thus in view of these suggestions and the fact that protons, either free or bound, are presumably produced during mitochondrial respiratory chain activity, it appeared important to study proton movements in respiring mitochondria *per se*.

Methods and Materials

Rat liver mitochondria were isolated by the method of Hogeboom (1955) as described by Myers and Slater

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[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; TMPD, tetramethyl-*p*-phenylenediamine; DPN, 2-4-dinitrophenol.

(1957). The mitochondrial suspension, 35–45 mg of protein/ml, was stored at 0° and used within 4 hr. Mitochondrial protein was determined by the biuret method as described by Cleland and Slater (1953).

Oxygen utilization and H^+ concentration were measured simultaneously in a small water-jacketed reaction cell which was maintained at 30° and stirred with a magnetic stirrer. The volume of the reaction vessel was 2 ml and this volume completely filled the vessel. The electrodes passed through a tapered Teflon stopper which also contained a small hole (3.5-mm diameter) through which additions were made with 10-μl microsyringes.

Oxygen utilization was measured polarographically using a small Clark-type oxygen electrode (Titron Instrument Co., Sandringham, Australia) in conjunction with an Oxygraph, Model K (Gilson Medical Electronics, Wis.). The electrode was covered with 6 μ of Teflon membrane, a gift from Dr. I. A. Silver, which allowed a rapid response time (*ca.* 3 sec). Buffer solutions were calibrated for oxygen content according to the method of Chappell (1964).

H^+ concentration was measured with a small (4-mm o.d.) combination pH electrode (Titron Instrument Co., Sandringham, Australia) connected to a Radiometer pH meter, Model 27 (Radiometer, Copenhagen, Denmark). The electrolyte used in the electrode was saturated NaCl and the flow rate through the junction was only 1–2 μl/hr. The output voltage of the pH meter was first passed to a Rikadenki preamplifier, Model A-10 (Rikadenki Kogyo Co. Ltd., Tokyo, Japan), and then to a Heath servo-recorder, Model EUW-20 AE (Heath Co., Benton Harbour, Mich.). A suitable resistance network was used to match the output of the pH meter with the preamplifier over the desired range and a suitable backing-off circuit enabled a pH change of anywhere between 0.01 and 1.0 unit to give a full scale deflection on the recorder, starting at any initial pH value in the range 7.0–7.6. Changes in H^+ concentration were not determined by reference to the pH in view of the varying buffer capacities of the different media used and the suspension errors mentioned by Bartley and Davies (1954) which were found to be considerable in some of the weakly buffered media. Instead H^+ concentration was determined directly by reference to the recorder deflection caused by small additions of hydrochloric acid (3–10 μl of 50 mM HCl) at the end of each experiment, according to the method described by Nishimura *et al.* (1962). A weakly buffered medium composed of 0.17 M sucrose and 40 mM choline chloride described by Chappell and Crofts (1965) was found to be most satisfactory for recording small changes in H^+ concentration. In this medium some additions caused artefacts in the recording, as determined by control experiments with no mitochondria, and where these occurred a break has been left in the trace illustrated.

Ferricyanide reduction was followed in a Shimadzu recording spectrophotometer, Model SV 50 A (Seisakusho Ltd., Kyoto, Japan), at 420 mμ and 30°. The author is indebted to the C. S. I. R. O., Division of Animal Health, Parkville, Australia, for the use of this instrument. Incubation medium plus mitochondria were placed in both the reference and sample cuvets and the reaction was started by the addition of 15 μl of freshly prepared 0.2 M potassium ferricyanide to the sample cuvet.

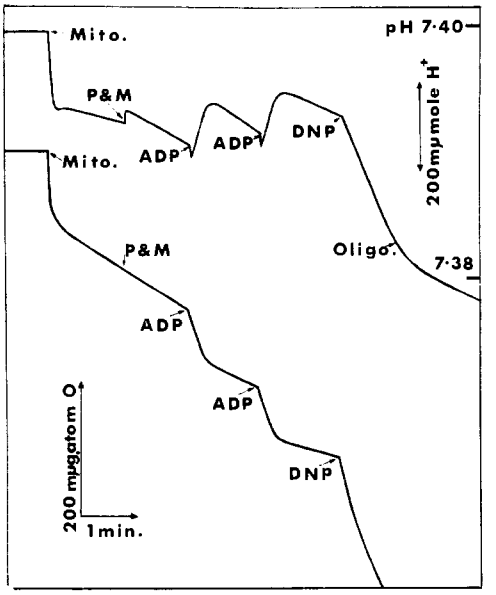

FIGURE 1: The effect of various additions on oxygen uptake and H^+ concentration in respiring preparations of rat liver mitochondria. The incubation mixture contained 50 mM sucrose, 130 mM KCl, 7.5 mM $MgCl_2$, and 30 mM sodium potassium phosphate buffer, pH 7.40, in a volume of 2.0 ml; temperature 30°. Additions in the order indicated were, mitochondria (equivalent to 4.2 mg of protein), 2 mM Tris–pyruvate plus 2 mM Tris–malate, 183 mμmoles of ADP (the same amount in both additions), 5×10^{-5} M 2,4-DNP, and oligomycin (equivalent to 2.2 μg/mg of mitochondrial protein). The upper trace represents the recording of H^+ concentration, a downward deflection indicating an increase in concentration. The lower trace represents the recording of oxygen concentration, a downward deflection indicating an uptake of oxygen.

Reagents. ADP was purchased from the Sigma Chemical Co. and the actual ADP content was determined by the method of Slater (1953). Antimycin A was purchased from the Kyowa Fermentation Co., Tokyo, Japan, and the oligomycin was a gift from the Upjohn Co., Mich. All substrates were AR grade acids which were neutralized with Tris.

Results

Response and Accuracy of the Apparatus for Recording Small Changes in H^+ Concentration. It seemed desirable to assess the response of the apparatus to various additions and changes in mitochondrial respiration and to determine the accuracy of the recording system at high amplification. Since during the formation of ATP from

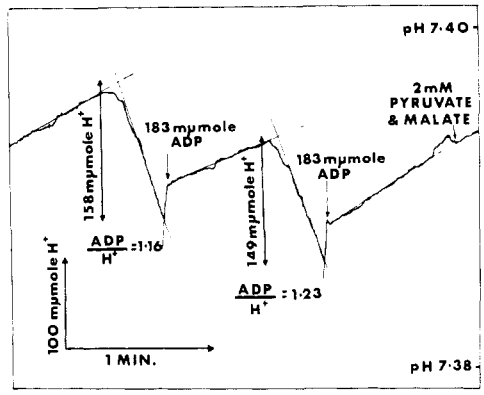

FIGURE 2: The effect of ADP on H^+ concentration in a phosphorylating preparation of rat liver mitochondria. The figure shows an actual recorded trace with tangents drawn on the trace subsequently; the intersection of these tangents indicates the exact point at which the decrease in H^+ concentration, caused by the ADP additions, ceases. Other experimental conditions as in Figure 1.

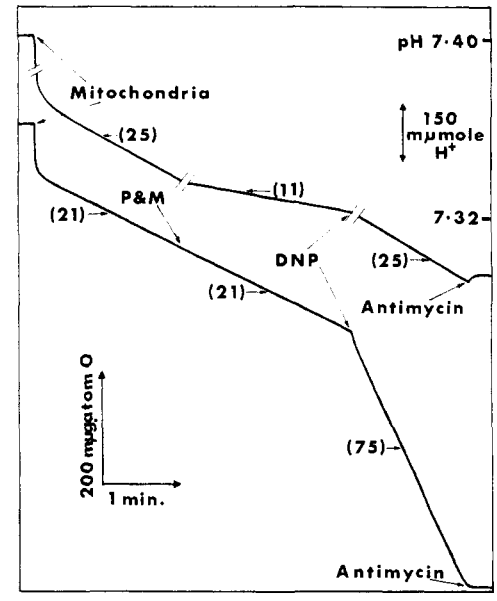

FIGURE 3: The effect on DNP and antimycin on oxygen uptake and H^+ concentration in a respiring preparation of rat liver mitochondria. The incubation medium contained 0.17 M sucrose and 40 mM choline chloride adjusted to pH 7.4 with Tris; temperature 30°. Additions in the order indicated were, mitochondria (equivalent to 4.4 mg of protein), 2 mM Tris–pyruvate plus 2 mM Tris–malate, 5×10^{-5} M 2,4-DNP, and antimycin (2.1 μg/mg of mitochondrial protein). The traces are as described in Figure 1. The figures in brackets in relation to the upper trace represent the rate of increase in H^+ concentration in millimicromoles of H^+ per minute per milligram of mitochondrial protein and in relation to the bottom trace represent rates of oxygen uptake in millimicrogram-atoms of oxygen per minute per milligram of mitochondrial protein.

ADP there is an uptake of H^+ ions according to the equation

$$ADP + P_i + nH^+ \rightleftharpoons ATP + H_2O \qquad (1)$$

it was decided to examine the changes in H^+ concentration of respiring rat liver mitochondrial preparations in response to small additions of ADP in a medium capable of supporting phosphorylation. Nishimura *et al.* (1962) have calculated theoretically the value of n, which equals $\Delta H^+/\Delta P_i$ in eq 1, as 0.851 at pH 7.4, assuming magnesium complexes of the adenine nucleotides, and 0.891 assuming no such complexes.

The results shown in Figure 1 indicate clearly that there is a decrease in H^+ concentration which is initiated by the addition of ADP and continues while the ADP is phosphorylated (*cf.* Swanson, 1957). The decrease in H^+ concentration ceases at exactly the same time as all the ADP is phosphorylated, this point being indicated by the cutoff point in the oxygen trace. This is followed by a reversion to the slower increase in H^+ concentration which is dealt with in the next section. The exact point at which the decrease in H^+ concentration ceased was determined by the intersection of tangents drawn on the trace as shown in Figure 2. This method is analogous to the one used by Chance and Williams (1955) to determine the exact amount of oxygen utilized during the phosphorylation of a small amount of added ADP.

Values for n, here $\Delta H^+/\Delta ADP$, at pH 7.40 were determined for three different substrates, *i.e.*, pyruvate plus malate, succinate, and glutamate (15 estimations in all). The average value of n was found to be 0.878 with a standard deviation of 0.066, which is in excellent agreement with the results of Nishimura *et al.* (1962) who found an experimental value of 0.882, with a standard deviation of 0.071, for photophosphorylation in *Rhodospirillum rubrum* chromatophores.

Thus it appears from the results shown in Figures 1 and 2 and the above calculations that the apparatus is capable of responding to rapid changes in H^+ concentration and that the recorded changes are quite accurate at high amplification.

Proton Extrusion from Mitochondria during Respiration. The results of a typical experiment are shown in Figure 3 and show that the H^+ concentration of the medium increased at a constant rate when rat liver mitochondria were added to 0.17 M sucrose and 40 mM choline chloride at 30°. The rate was decreased by the addition of pyruvate plus malate and increased by the subsequent addition of 2,4-DNP. The addition of antimycin immediately stopped the increase in H^+ concentration. As the increase in H^+ concentration occurs only on the addition of the mitochondria it is considered that this represents an extrusion of protons from the mitochondria. In the experiment shown in Figure 3 the rate of proton extrusion was 11 mμmoles of H^+/min

per mg of mitochondrial protein when the substrate oxidized was pyruvate plus malate while the rate of oxygen uptake was 21 mμg-atoms/min per mg of mitochondrial protein. If two electrons are transferred per oxygen atom utilized, the rate of the proton extrusion is *ca.* 25% of the rate of electron transport. In different experiments in various media the rate of proton extrusion was found to vary between 25 and 70% of the rate of electron transport with pyruvate plus malate as substrate.

A. THE EFFECT OF VARIOUS INCUBATION MEDIA. The 0.17 M sucrose plus 40 mM choline chloride medium of Chappell and Crofts (1965) was found to be the most satisfactory medium for recording small changes in H^+ concentration as with this medium respiratory rates remained linear and there was no evidence of mitochondrial swelling. While changes similar to those shown in Figure 3 were recorded in unbuffered sucrose there was too much drift in the pH to allow accurate readings. The results obtained in a medium containing 50 mM sucrose, 130 mM KCl, 7.5 mM $MgCl_2$, and 30 mM sodium potassium phosphate, pH 7.4, as shown in Figure 1, were also similar. However, the effects of uncoupling agents on the rate of proton extrusion were complex and are considered in a later section.

B. THE EFFECT OF ADDED CATIONS. It is important to note that in the type of experiment shown in Figure 3 there are no added cations (with the exception of choline ions) in the incubation medium. The addition of 1.5 mM $MgCl_2$ plus 1.5 mM sodium phosphate, pH 7.4, or 6.5 mM KCl had virtually no effect on the rate of proton extrusion.

C. THE EFFECT OF VARIOUS SUBSTRATES. While the addition of pyruvate plus malate or glutamate had little effect on the rate of respiration, both reduced the rate of proton extrusion. The addition of succinate caused a greater reduction in the rate of proton extrusion, but at the same time slightly increased the rate of respiration (Table I).

TABLE I: A Comparison between Rates of Oxygen Uptake and Proton Extrusion in Preparations of Rat Liver Mitochondria Oxidizing Different Substrates.[a]

Substrate	Oxygen Uptake (mμg-atoms/min mg of protein)	Proton Extrusion (mμmoles/min mg of protein)
Endogenous	14.0	16.7
2 mM glutamate	14.0	9.8
2 mM succinate	22.0	3.9
2 mM pyruvate plus 2 mM malate	13.9	9.7
2 mM pyruvate plus 2 mM malate plus 5×10^{-5} M DNP	73.0	25.5

[a] Experimental conditions as described in Figure 3. The figures are the average of three experiments.

It was of considerable interest to know whether oxidation in the terminal region of the respiratory chain also supported proton extrusion. A combination of ascorbate and TMPD (N,N,N′,N′-tetramethyl-*p*-phenylenediamine), first introduced by Jacobs (1960), provides an efficient electron donor system at the cytochrome *c* level. However this electron donor system could not be used successfully in these experiments as the oxidation of ascorbate is accompanied by a decrease in H^+ concentration due to the formation of dehydroascorbate and the consequent loss of the acidic enol group at position 3 of the molecule. This was demonstrated experimentally as the addition of antimycin, which blocked endogenous respiration and its associated proton extrusion, actually enhanced the rate of decrease in H^+ concentration with ascorbate and TMPD as substrate.

D. THE EFFECT OF RESPIRATORY INHIBITORS. Both antimycin A (at *ca.* 1 μg/mg of mitochondrial protein) and cyanide, 10^{-2} M, prevented the extrusion of protons immediately. Anaerobiosis had a similar effect. The phosphorylation inhibitor, oligomycin, had no noticeable effect on the rate of extrusion, except in special circumstances as mentioned below.

E. THE EFFECT OF UNCOUPLING AGENTS. A variety of uncoupling agents, *viz.*, 5×10^{-5} M 2,4-DNP, 10^{-5} M dicoumarol, and sodium oleate (20 μg/mg of mitochondrial protein), all considerably enhanced the rate of proton extrusion. Triton-X 100 at a concentration of 9.0 μg/mg of mitochondrial protein completely stopped the proton extrusion while respiration was increased slightly. The effect of 2,4-DNP is shown in Figure 3. The addition of DNP increased the rate of proton extrusion some 2.5-fold. However, although these uncoupling agents markedly increased the respiration rate (in the order of five–sixfold) the rate of proton extrusion compared with the rate of electron transport was significantly less in all cases than it was in the absence of the uncoupling agent. Considering the results shown in Figure 3, the rate of proton extrusion is equivalent to *ca.* 17% of the rate of electron transport in the presence of DNP compared with 30% before the addition of the DNP (see above for calculations).

In experiments carried out in the phosphorylating medium, such as in Figure 1, if DNP or the other uncoupling agents were added to the reaction mixture after the ADP additions there was a very marked increase in the rate of proton extrusion, up to 20-fold in some cases. Because this increase was markedly inhibited by oligomycin (Figure 1) and was not completely inhibited by antimycin it is probable that it was due mainly to the induced breakdown of preformed ATP and the consequent release of protons by a reversal of the reaction shown in eq 1. The increase in the rate of proton extrusion caused by the addition of the uncoupling agent was not nearly so marked if ADP had not been added previously. In this case oligomycin had

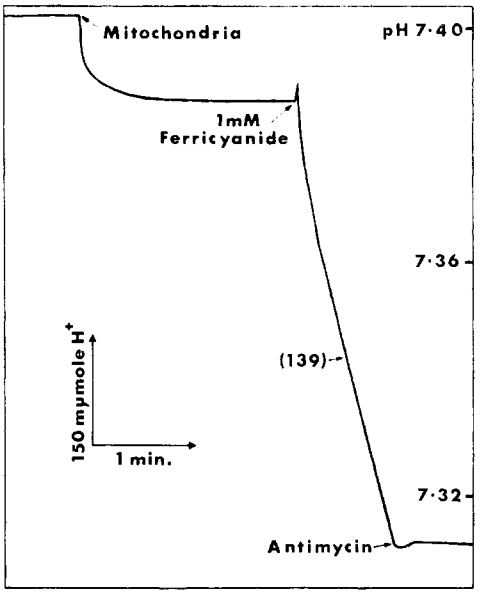

FIGURE 4: The effect of ferricyanide and antimycin on H^+ concentration in a respiring preparation of rat liver mitochondria. The incubation mixture contained 0.22 M sucrose, 25 mM Tris–chloride (pH 7.4), 10 mM KCN, and 2 mM Tris–succinate in a volume of 2 ml. Additions in the order indicated were, mitochondria (equivalent to 4.0 mg of protein), 1 mM potassium ferricyanide, and antimycin (2.5 μg/mg of mitochondrial protein). The figure in brackets represents the rate in decrease in H^+ concentration in millimicromoles per minute per milligram of mitochondrial protein.

little effect and antimycin completely stopped the proton extrusion.

F. THE EFFECT OF CYANIDE PLUS FERRICYANIDE. Ferricyanide may be used as an alternative electron acceptor to oxygen in the presence of cyanide, and electrons are accepted predominantly at the cytochrome *c* level (Estabrook, 1961). The results shown in Figure 4 indicate that the addition of mitochondria to a medium containing sucrose, Tris–chloride (pH 7.4), succinate, and 10^{-2} M cyanide caused a slight drop in pH but there was no extrusion of protons after the initial equilibration period. The addition of 1 mM ferricyanide caused a very rapid extrusion of protons which, after a short time, reached a constant rate of 139 mμmoles of H^+/min per mg of mitochondrial protein. The extrusion was completely stopped by the addition of antimycin. The experiment was repeated immediately, the rate of ferricyanide reduction being determined in a recording spectrophotometer. It was 147 mμmoles of ferricyanide reduced/min per mg of mitochondrial protein. Thus the rate of proton extrusion was 95% of the rate of electron transport, since ferricyanide is a one-electron acceptor. In a number of other experiments carried out under these conditions it was found that the rate of proton extrusion was approximately equal to the rate of electron transport.

Discussion

The results presented in this paper indicate that there is a constant increase in H^+ concentration of the medium when rat liver mitochondria are respiring. Similar observations have been made by other workers (see Figure 8 of Pressman, 1963, and Figure 2 of Brierley *et al.*, 1964). However, because of the preoccupation of these workers with other facets of mitochondrial ion movements these observations were not pursued. As the increase in H^+ concentration only occurs following the addition of mitochondria it is considered that this phenomenon represents an extrusion of protons from the mitochondria. A similar conclusion was very recently reached by Mitchell and Moyle (1965a) as a result of their ingenious experiments involving pH measurements following the addition of small amounts of oxygen to anaerobic suspensions of rat liver mitochondria. This concept of a respiration-dependent extrusion of protons then raises the question as to what is the source of protons within the mitochondria?

First, H^+ ions could be released by the breakdown of endogenous ATP according to eq 1. Koivusalo and Slater (1966) have recently determined the amount of reactive high-energy intermediates initially present in rat liver mitochondria to be equivalent to 0.20 μmole/mg of protein. If one assumed this is all in the form of ATP, or can be converted to ATP, then there are some 200 mμmoles of ATP/mg of protein which could give rise to an equivalent amount of H^+. This would be sufficient to maintain a rate of proton extrusion of 10 mμmoles of H^+/min per mg of protein for 20 min. It is, however, unlikely that ATP would break down during active respiration. Also, in experiments not recorded here the proton extrusion was found to continue for 1 hr or more providing respiration continued. Furthermore, if ATP breakdown were the source of protons one would expect oligomycin to inhibit this extrusion, which it does not. Also, it is difficult to see why the addition of a respiratory inhibitor, such as antimycin, should immediately stop proton extrusion. Indeed one would expect that if respiration were blocked, the rate of breakdown of endogenous ATP would increase, *i.e.*, native ATPase activity, and hence proton extrusion would be stimulated under these conditions (see Ter Welle and Slater, 1964).

Second, protons could be released from the mitochondria as other ions are taken up in an ion exchange reaction as indeed has been reported in a number of cases (Brierley *et al.* 1962; Chappell and Greville, 1963; Chance, 1965). However, in the experiments in sucrose–choline chloride medium described here there is no obvious source of cations in the medium for exchange reactions. Choline would be present in the form of a complex organic cation but the exchange reactions demonstrated have involved simple monovalent and divalent cations. Also the release of protons was observed when the mitochondria respired in 0.25 M sucrose although the actual measurement of the rates of proton extrusion were unreliable in this medium. Furthermore, the addition of ions such as Mg^{2+}, $H_2PO_4^-$, and K^+,

which have been shown to be involved in mitochondrial exchange reactions for protons (Brierley *et al.*, 1962; Moore and Pressman, 1964), had little or no effect on the rate of proton extrusion. Also in the experiments described here the addition of uncoupling agents increased the rate of proton extrusion, whereas the uptake of cations and the consequent release of protons is completely prevented by uncoupling agents (Lehninger, 1964). Finally, in reported experiments where protons were released from the mitochondria as a result of exchange with other ions, the subsequent addition of an uncoupling agent or antimycin caused an immediate increase in pH of the incubation medium as protons reentered the mitochondria and the previously absorbed cations were expelled (*e.g.*, see Moore and Pressman, 1964). In the present experiments, the addition of an uncoupling agent or antimycin, following a period in which there was a steady decrease in pH, was not accompanied by an increase in pH. The addition of antimycin, *e.g.*, merely stopped the decrease in pH. Thus the respiration-dependent extrusion of protons observed here would not appear to be due to the release of protons by an ion-exchange mechanism. However, there is the possibility that loosely bound cations such as magnesium and also phosphate may give rise to exchange reactions and a secondary movement of protons.

A third possible method by which protons may be produced in the mitochondria is directly as a product of electron transport. Mitchell (1963) has been a keen proponent of such a mechanism as providing the appropriate potential across the mitochondrial membrane to drive the formation of ATP. The results presented here certainly indicate that the proton extrusion is dependent on respiration as it is immediately stopped by the addition of antimycin or cyanide. In recent experiments Mitchell and Moyle (1965a) have demonstrated the respiration-dependent release of protons from mitochondria. Also in the present experiments, in the presence of cyanide and ferricyanide, the rate of proton extrusion was virtually the same as the rate of electron transport with succinate as substrate. The oxidation of substrates like glutamate and pyruvate plus malate, which should give rise to three protons per pair of electrons transferred during respiration, was associated with a greater rate of proton extrusion than the oxidation of succinate which gives rise to only two protons per pair of electrons. These latter findings are of particular interest in view of the recent experimental findings of Mitchell and Moyle (1965a). They found $\Delta H^+/O$ (*i.e.*, protons released per oxygen utilized) for succinate oxidation of four, and six for the oxidation of β-hydroxybutyrate.

Thus it appears that the most logical explanation of the respiration-dependent proton extrusion reported here is that protons are produced as a direct product of electron transport. A similar conclusion has recently been reached by Mitchell and Moyle (1965a) as a result of extensive studies on proton release associated with the uptake of small amounts of oxygen in rat liver mitochondria. Mitchell and Moyle (1965b) have provided additional support for this conclusion with their important findings with phosphorylating submitochondrial particles in which the respiration-dependent proton flow was in the reverse direction. Thus the concept of a H^+ pump mechanism, as suggested by Chappell and Crofts (1965), is now supported by some experimental evidence. Whether such a mechanism provides the underlying driving force for all mitochondrial ion transport, as also suggested by these authors, remains to be seen. Also whether this mechanism provides the basic electrochemical potential to generate ATP as suggested by Mitchell (1963) requires further investigation. The fact that in the present experiments the addition of ADP caused a halt in the proton extrusion which resumed immediately when all the ADP was phosphorylated is inconclusive. The very rapid uptake of protons during the phosphorylation of ADP (eq 1) may have merely masked a continuing slow rate of proton extrusion, or the extrusion may have stopped while the ADP was phosphorylated. This point requires further investigation.

Acknowledgments

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The Amino Acid Sequence of *Clostridium pasteurianum* Ferredoxin*

Masaru Tanaka, Tadayoshi Nakashima, Ann Benson, Howard Mower, and Kerry T. Yasunobu†

ABSTRACT: Four derivatives of the *Clostridium pasteurianum* ferredoxin were prepared from trichloroacetic acid treated ferredoxin. They were hydrolyzed with chymotrypsin, trypsin, or pepsin and the peptides were purified by chromatography on Dowex 50-×2 and AG 1-×2.

The peptides thus obtained were further purified by paper chromatography, paper electrophoresis, or column chromatography. The sequences of the purified peptides were determined by the Edman degradative method, dinitrophenylation, by the use of leucine aminopeptidase and carboxypeptidase A and B. From the sequences of the individual peptides and the overlaps between them, the total amino acid sequence of the 55 amino acid residues comprising the *C. pasteurianum* ferredoxin was deduced.

Ferredoxin, a nonheme iron containing protein was isolated from *Clostridium pasteurianum* by Mortenson *et al.* (1962, 1963). The electron transport protein has also been isolated from numerous anaerobic bacteria in crystalline form. A recent review article (Valentine, 1964) discusses the occurrence, structure, and function of the various bacterial ferredoxins.

The latest report on the physicochemical properties of *C. pasteurianum* ferredoxin indicates that the protein has a molecular weight of *ca.* 6000, contains about 50 amino acid residues, and has seven iron atoms per molecule of protein (Lovenberg *et al.*, 1963). The protein contains no tryptophan, methionine, or –S–S– bridges. Our laboratory has published a preliminary note on the amino acid sequence of the protein (Tanaka *et al.*, 1964b).

Experimental Section

Preparation of Ferredoxin. The procedure for isolating ferredoxin from *C. pasteurianum* was based upon a method devised by Mortenson *et al.* (1962, 1963) and has been described in a previous publication (Tanaka *et al.*, 1964b). For purity check, each preparation of ferredoxin was analyzed for the 390:280 mμ ratio (0.79 or greater), the amino acid composition, and the NH_2-terminal amino acid residue.

Derivatives of Ferredoxin. Iron and sulfide were removed from intact ferredoxin by trichloroacetic acid precipitation as previously described (Tanaka *et al.*, 1964b). This method is a simple and easy procedure for obtaining iron-removed and sulfide-free ferredoxin (TCAFd)[1] without cleavage of the peptide bonds.

Due to scarcity in the protein of peptide bonds which are hydrolyzed by trypsin or chymotrypsin, and due to the high content of cysteine residues, the ferredoxin molecule posed certain problems. To overcome these difficulties, four derivatives were prepared.

A. OXIDIZED FERREDOXIN (OFd).[1] This was made in the usual manner by performic acid oxidation of the trichloroacetic acid treated ferredoxin (TCAFd) (Schram *et al.*, 1954). After oxidation, the oxidized ferredoxin was passed through a column of Sephadex G-25 and then lyophilized. The oxidized ferredoxin obtained was a flocculent and white powder which was water soluble.

B. *S*-β-AMINOETHYLCYSTEINYL-FERREDOXIN (AE-

* From the Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii. *Received November 24, 1965; revised January 17, 1966.* Supported by a research grant (GB 1363) from the National Science Foundation.

† To whom requests for reprints should be addressed.

[1] The following abbreviations were adopted: OFd, oxidized ferredoxin; AECFd, *S*-β-aminoethylcysteinyl-ferredoxin; TCAFd, trichloroacetic acid treated ferredoxin; CAMCFd, *S*-carboxamidomethylcysteinyl-ferredoxin; DECFd, *S*-β-dithiocarbaminoethylcysteinyl-ferredoxin; BAW, butanol–acetic acid–water; BPAW, butanol–pyridine–acetic acid–water; BPW, 1-butanol–pyridine–water; LAP, leucine aminopeptidase; CPase, carboxypeptidase; AEC, *S*-β-aminoethylcysteine; PTH, phenylthiohydantoin; and PTC-, phenylthiocarbamyl.