to Oscar Mayer and Company for the gift of slaughterhouse material and to Mr. A. D. HEINDEL for the supervision of the large scale preparation of mitochondria.

SUMMARY

A method for the extraction of soluble succinic dehydrogenase from the particulate succinic dehydrogenase complex (SDC) is described.

The enzyme, as extracted from SDC, catalyzes the dehydrogenation at 38° of 10 μmoles of succinate per min per mg with ferricyanide as the electron acceptor, or 14 μmoles of succinate per min per mg with phenazine methosulfate as electron acceptor. It contains $3 - 5 \cdot 10^{-3}$ μmoles of flavin as well as $5 - 10 \cdot 10^{-3}$ μg atoms of non-heme iron per mg of protein.

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STUDIES ON THE ELECTRON TRANSPORT SYSTEM

IX. FRAGMENTATION OF DPNH OXIDASE*

BRUCE MACKLER** AND NATHAR PENN***

Institute for Enzyme Research, University of Wisconsin, Madison, Wis. (U.S.A.)

In a previous communication[1] we have described the effects of deoxycholate on the DPNH oxidase (reduced diphosphopyridine nucleotide oxidase)—a derivative form of the electron transport particle which has lost succinic dehydrogenase activity. DPNH oxidase is prepared in a "closed" state in which external cytochrome *c* cannot interact with the heme chain either as electron acceptor or donor. After exposure to deoxycholate the oxidase is converted to an open state in which cytochrome *c* can react maximally with the heme chain, whereas DPNH oxidase in its closed form cata-

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lyzes the oxidation of DPNH by oxygen in absence of added cytochrome *c* and this capacity is abolished after the particle has been exposed to deoxycholate. In the present communication evidence is presented that cholate-ammonium sulfate mixtures which can duplicate the opening phenomenon fragment DPNH oxidase into two particles and that the "opening phenomenon" can be in part interpreted in terms of this fragmentation. One of the derivative particles (red fraction) contains DPNH dehydrogenase and cytochromes *b* and c_1, while the second particle (green fraction) contains cytochrome oxidase and cytochromes *a* and a_3. The optimum conditions for the isolation of the red fraction are not the same as those for the isolation of the green fraction although both are found under either set of conditions.

EXPERIMENTAL

Methods and reagents

Assays of enzymic activity and determinations of flavin, copper, iron and protein have been described previously[1,2].

The sources of the following reagents were respectively: DPNH and cytochrome *c* (Sigma Chemical Co.); "1:300" trypsin (Nutritional Biochemicals Corp.); and bovine serum albumin (Armour and Co.).

Preparation of particles

1. *Red particle fraction.* A suspension of DPNH oxidase in 5% sucrose (20 mg per ml), prepared as described previously[1], was treated with 1:300 trypsin* (0.02 mg per mg of protein) and neutral sodium cholate (0.4 mg per mg of protein). The mixture was incubated for 30 min at 23° and then cooled rapidly to 0°. All subsequent procedures were carried out in the temperature range 0 to 2°. Solid ammonium sulfate (23 g per 100 ml of suspension) was then added. The mixture was stirred gently for 5 min and then centrifuged in the number 40 rotor of the Spinco preparative ultracentrifuge for 10 min at 40,000 r.p.m. The residue was discarded. The red-brown supernatant was treated with a saturated ammonium sulfate solution (neutral) until definite cloudiness developed and centrifuged as before. The sediment (fraction 1) was suspended in a minimal volume of a solution which was 30% with respect to sucrose and 1.5% with respect to bovine serum albumin. The supernate was treated again as above with neutral ammonium sulfate solution (over the range of 40 to 70% saturation). In this way three further sediments, all deep red (fractions 2, 3 and 4), were obtained and these were also suspended in the sucrose albumin mixture. Fraction 4 was inactive enzymically. Fraction 1 was contaminated slightly with cytochrome oxidase activity, whereas fractions 2 and 3 which had high DPNH cytochrome *c* reductase activity showed essentially no cytochrome oxidase activity. Fractions 2 and 3 retained full activity when stored at —20° for 1 to 2 weeks. After this period a definite decline in activity was observed.

The most active preparations of fractions 2 and 3 catalyzed the oxidation by cytochrome *c* of 2.5 μmoles of DPNH per min per mg protein at 38°. This represents an activity which is about 2 times higher than that of the starting material (assayed after "opening" with deoxycholate). Fractions 2 and 3 account for 35% of the DPNH cytochrome *c* reductase activity of the starting suspension of DPNH oxidase.

* The presence of trypsin is not essential for the fragmentation of DPNH oxidase. However, the fractionation with cholate ammonium sulfate appears to proceed more sharply in presence of the trypsin preparation.

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2. *Green particle fraction.* A suspension of DPNH oxidase (20 mg protein per ml) was treated with 1:300 trypsin (0.025 mg per mg protein) and neutral sodium cholate (1.3 mg per mg protein). The mixture was incubated for 80 min at 23°, cooled to 0° and then fractionated with ammonium sulfate as described above. At the higher cholate:protein ratio the green particle was extracted while the red particle remained in the sediment. After the residue at 40% saturation of ammonium sulfate was removed, the supernatant was then fractionated with ammonium sulfate over the range of 40 to 70% saturation. Four fractions (1–4) were obtained. The sedimented particles were suspended in 30% sucrose. Fractions 2, 3 and 4 (deep green suspensions) showed high and virtually equal cytochrome oxidase activity, but essentially no DPNH cytochrome *c* reductase activity. However, there were residual traces of DPNH dehydrogenase activity (2,6-dichlorophenol indophenol as acceptor). The most active preparations catalyzed the oxidation of 24 μmoles of reduced cytochrome *c* per min per mg at 38°. This specific activity represents a 4-fold concentration of activity over that of the starting suspension of DPNH oxidase (after "opening" with deoxycholate). Preparations were stored for 3 weeks at —20° without loss of activity. Approximately 40% of the original cytochrome oxidase activity of the starting suspension of DPNH oxidase was recovered in fractions 2 to 4.

RESULTS

Spectroscopic and enzymic properties

1. *Red particle fraction.* When a suspension of the red particle fraction was reduced with an excess of DPNH or with dithionite and the light absorption of the reduced form was measured against that of the oxidized form as blank, difference spectra such as are shown in Fig. 1 were obtained. The spectra show absorption maxima at 562 to 563, 553, 532, 525 and 428 mμ, indicating the presence of cytochrom es*b* and c_1. The absorption maxima characteristic of cytochromes *a* and a_3 (605 and 444 mμ respectively) were absent. As shown in Fig. 1 approximately 80% of cytochrome c_1 and 60% of cytochrome *b* were reduced by DPNH. The spectrum of the reduced pyridine hemochromogens prepared as described previously[3] showed absorption maxima only at 420, 525 and 555 mμ. The presence of cytochromes *a* and a_3 in the red particle fraction is thus ruled out.

The effects of the addition of increasing amounts of DPNH on the degree of reduction of cytochromes c_1 and *b* are shown in Fig. 2. As shown in the figure, cytochrome c_1 became reduced at levels of DPNH that did not reduce cytochrome *b*. As the concentration of DPNH was increased (above the concentration necessary to fully reduce c_1) a gradual reduction of cytochrome *b* occurred.

DPNH cytochrome *c* reductase activity was measured routinely in 0.04 *M* phosphate buffer (in presence of 0.01 *M* azide to suppress any residual enzymic oxidation of reduced cytochrome *c*). Activity was maximal over the pH range 7.5 to 8.5. Phosphate ions were not essential for activity. Other buffers such as tris (hydroxymethyl) aminomethane (hereinafter referred to as "tris"), histidine, and inorganic pyrophosphate, were equally satisfactory.

Antimycin A at low levels[2] and amytal (2.0 m*M*) inhibited cytochrome *c* reductase activity 95 to 100%. No preincubation of the particle suspension with inhibitor

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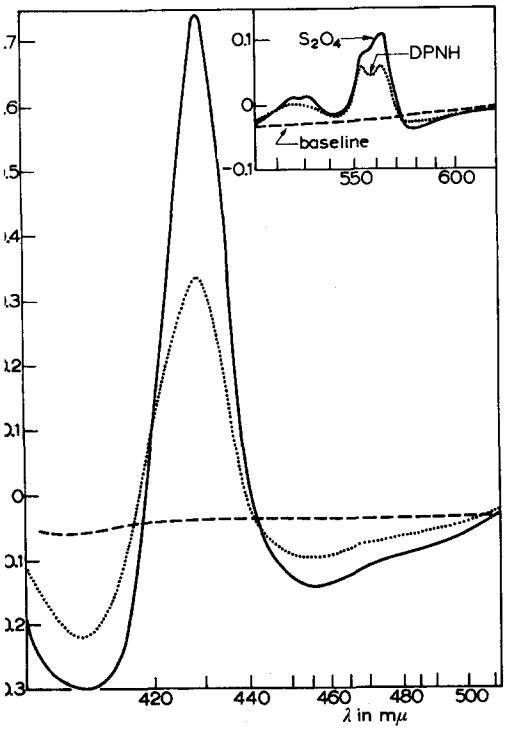

Fig. 1. Difference spectra (reduced minus oxidized) of the red fraction. The enzyme suspension was reduced with dithionite (0.2 mg) or DPNH (1 mg). The sample contained 4.6 mg of enzyme protein in 1 ml of 0.04 *M* phosphate buffer (pH 7.5).

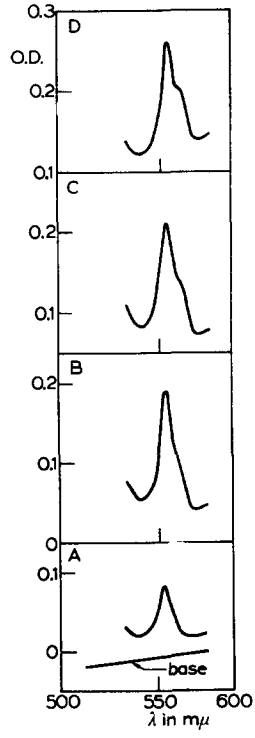

Fig. 2. Extent of reduction of the hemes of the red particle with increasing levels of DPNH: (a) 0.00447 μmole, (b) 0.0134 μmole, (c) 0.0313 μmole and (d) 0.0581 μmole. The cuvette contained 7 mg of enzyme protein in a total volume of 1 ml of 0.04 *M* phosphate buffer of pH 7.5. The reaction was carried out at 23°.

was necessary. The diaphorase activity of the particle suspension (reduction of 2,6-dichlorophenolindophenol or ferricyanide with DPNH as substrate) was not affected by either antimycin or amytal in concentrations 5 times higher than those necessary to inhibit cytochrome *c* reduction completely. $1 \cdot 10^{-3}$ *M* *o*-phenanthroline inhibited cytochrome *c* reductase activity 80 to 90% while $1 \cdot 10^{-3}$ *M* Versene, $1 \cdot 10^{-3}$ *M* Versene EDG, $1 \cdot 10^{-2}$ *M* azide and $1 \cdot 10^{-4}$ *M* *p*-chloromercuribenzoate were without effect on this activity.

The Michaelis constants of the red particle fraction for DPNH and cytochrome *c* are $1.2 \cdot 10^{-5}$ and $2.2 \cdot 10^{-6}$ *M* respectively.

2. *Green particle fraction.* Fig. 3 shows the difference spectrum (reduced with dithionite minus oxidized) of a suspension of the green particle fraction. The presence of cytochromes *a* and a_3 is indicated by the absorption bands at 605 and 444 mμ respectively. There was no spectroscopic evidence for the presence of cytochromes *b* and c_1, characteristic of the red particle fraction. When the hemes of the green particle fraction are converted to the form of their pyridine hemochromogens the spectrum shows only two maxima at 432 and 590 mμ respectively, which are characteristic of the pyridine hemochromogen of cytochromes *a* and a_3.

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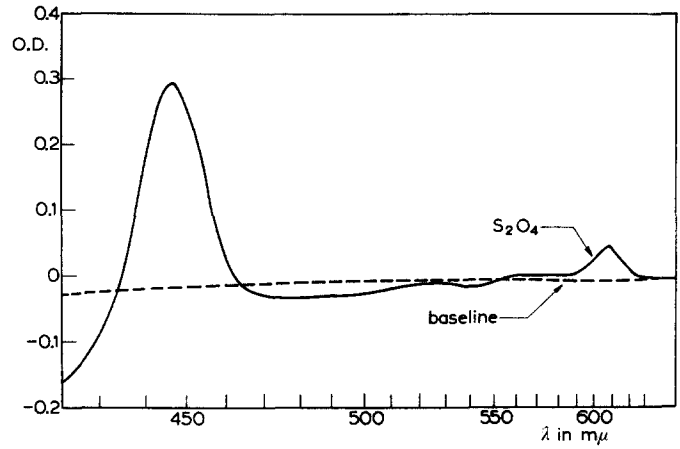

Fig. 3. Difference spectrum (reduced with dithionite minus oxidized) of the green particle fraction. Each cuvette contained 1.06 mg of protein in 1 ml of 0.04 phosphate buffer (pH 7.5).

The oxidation of reduced cytochrome *c* by oxygen was measured spectrophotometrically in cuvettes maintained at 38° and exposed to air. There was no increase in velocity when air was replaced by oxygen. Half-maximal velocity was attained in the oxidation when the concentration of cytochrome *c* was $3.6 \cdot 10^{-5}$ *M*. The assay system contained 0.04 *M* phosphate buffer at pH 7.5. There was no difference in rate over the pH range 6.5 to 7.5 in phosphate buffer. Phosphate is not essential for cytochrome oxidase activity since it may be replaced by tris or glycyl glycine. The oxidized form of cytochrome *c* in high levels (up to $1 \cdot 10^{-4}$ *M*) had no influence on the enzymic oxidation.

Cytochrome oxidase activity was completely abolished by cyanide ($1 \cdot 10^{-3}$ *M*), hydrogen sulfide ($1 \cdot 10^{-3}$ *M*) and azide ($1 \cdot 10^{-2}$ *M*). Versenol ($1 \cdot 10^{-3}$ *M*), Versene EDG ($1 \cdot 10^{-3}$ *M*), *p*-chloromercuribenzoate ($1 \cdot 10^{-4}$ *M*) and *o*-phenanthroline ($1 \cdot 10^{-3}$ *M*), had no effect on the rate of oxidation. Dithiodiethyl carbamate ($1 \cdot 10^{-3}$ *M*) progressively inhibited enzymic activity as the reaction proceeded. Inhibition developed 30 to 45 seconds after the reaction started and reached 75% by 2 minutes. Preincubation of the particle suspension (either in reduced or oxidized form) with the inhibitor did not change the pattern of inhibition.

Composition of the red and green particle fractions

In other communications we shall deal with the further purification of the particles carrying DPNH cytochrome *c* reductase and cytochrome oxidase activity respectively and with the composition of such purified particle preparations. For the purposes of the present communication only a few qualitative observations are reported. The parent particle, DPNH oxidase, contains flavin, non-heme iron, copper, cytochromes *a*, *b* and c_1* in the approximate molecular or atomic ratio of 1:15:6:2:2:2. When the parent particle is cleaved into the red and green derivative particles, the former contains the bulk of the flavin and all the cytochrome *b* and c_1, while the latter contains

* Cytochrome *c* is present in preparations of DPNH oxidase in variable amounts and since no definite correlation has yet been found between the amount of cytochrome *c* and the activity of DPNH oxidase, the cytochrome *c* content of the oxidase has not been referred to in the text.

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relatively little flavin and all the cytochrome *a*. Copper is concentrated exclusively in the green fraction while non-heme iron is distributed between the two fractions. The *a* heme and copper content of the green fraction is 4 times as high as that of the parent particle. This increase in concentration agrees with the four-fold concentration of cytochrome oxidase activity in the green fraction. The red particle fraction is probably a mixture of particles containing cytochromes *b* and c_1 with variable amounts of flavin. A considerable amount of flavin becomes liberated during the isolation proredure. This could explain the small degree of purification of the DPNH cytochrome *c* ceductase activity of the red fraction compared to the parent particle (about 2 times).

DISCUSSION

The observations reported above are consistent with the following interpretation of the opening phenomenon. If DPNH oxidase is represented by the formula

$$[F_D] — [bc_1]_2^* — [a + a_3]_2$$

where F_D represents the flavin of the DPNH dehydrogenase, *b*, c_1 and $a + a_3$, the corresponding cytochromes, and the bars between the brackets indicate that the components enclosed in the brackets are linked to the other components in the manner shown, then the fragmentation of this unit by cholate-ammonium sulfate may be described by the following scheme:

$$[F_D] — [bc_1]_2^* — [a + a_3]_2 \longrightarrow [F_D] — [bc_1]_2 + [a + a_3]_2$$

The continuous heme chain of the parent particle is cleaved and two derivative particles are formed each containing a different segment of the heme chain. Cytochrome *c* is needed for the interaction of the two daughter particles—undergoing reduction by DPNH when attached to the red particle and undergoing oxidation when attached to the green particle. In the original particle the heme chain is continuous, the bc_1 heme pairs being linked to the *a* hemes in an as yet unknown manner. Once the chain is disrupted and separate particles are formed, then only a compound like cytochrome *c* which can shuttle between the two now separate particles can reestablish electronic communication between the reducing and oxidizing segments of the heme chain. DPNH can be oxidized by oxygen in presence of the red and green particles and cytochrome *c* but not in the absence of cytochrome *c*.

WAINIO[4] and SMITH AND STOTZ[5] have already described fractions obtained by extraction of heart muscle with deoxycholate and cholate respectively which approximate closely the green fraction described above both in enzymic and spectroscopic properties. In a previous communication of this series CRANE AND GLENN[6] described the fragmentation of ETP into a red particle fraction with DPNH and succinic cytochrome *c* reductase activity and a green particle fraction with DPNH but no succinic dehydrogenase. This green particle catalyzes the oxidation of DPNH by oxygen when supplemented with cytochrome *c*.

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tissue used for preparation of mitochondria. The invaluable services of Mr. ALBERT HEINDEL in supervising the large-scale preparative work are gratefully acknowledged.

SUMMARY

DPNH oxidase has been fragmented with mixtures of cholate, ammonium sulfate and trypsin into a red particle with DPNH cytochrome *c* reductase activity (antimycin sensitive) and a green particle with cytochrome oxidase activity. This fragmentation probably accounts for the opening phenomenon in which the DPNH-oxidase activity of DPNH oxidase is abolished by exposure to deoxycholate, and two new activities emerge, *viz.* DPNH-cytochrome *c* reductase activity and ferrocytochrome *c* oxidase activity.

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A CRYSTALLINE TRYPSIN INHIBITOR FROM SWINE COLOSTRUM*

M. LASKOWSKI, BEATRICE KASSELL AND GLORIA HAGERTY

Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wis. (U.S.A.)

A recent study of the resistance to pepsin of six naturally occurring trypsin inhibitors[1] revealed striking differences. The two most resistant were a pancreatic inhibitor of KUNITZ AND NORTHROP[2] and bovine colostrum inhibitor[3,4]. Since it was previously postulated[3] that the physiological role of the inhibitor in colostrum is to protect the antibodies of colostrum from digestion by trypsin, it appeared desirable to extend our studies to the trypsin inhibitor of swine colostrum. Newborn pigs are known to be very sensitive to infection when deprived of normal colostrum. One might therefore expect swine colostrum to have a high level of inhibitor, highly resistant to pepsin, and this has proved to be the case.

The present paper describes the occurrence, purification and some of the properties of swine colostrum trypsin inhibitor.

EXPERIMENTAL

Material

The swine colostrum was collected in the Department of Animal Husbandry, University of Wisconsin, Madison, Wisconsin. We are greatly indebted to Prof. R. H. GRUMMER and Mr. H. C.

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