THE COENZYME Q REDUCTASE ACTIVITY OF DPNH-CYTOCHROME c REDUCTASE*

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Received May 9, 1967

We have recently reported (Singer and Salach, 1967; Salach et al., 1967) that, contrary to reports in literature (Pharo et al., 1966), DPNH-CoQ reductase (DPNH-ubiquinone reductase, Pharo et al., 1966) may be prepared from a variety of nonphosphorylating particles as well as from phosphorylating ones and that the preparations from these diverse sources are indistinguishable. DPNH-CoQ reductase may also be prepared by the exposure of DPNH dehydrogenase (Ringler et al., 1963), which is virtually devoid of CoQ_6 reductase activity, to 9% alcohol at pH 5.3 and 43°. The enzyme thereby loses its original properties and acquires high cytochrome c and CoQ reductase activities and becomes sensitive to inhibition by amytal and rotenone (Singer and Salach, 1967). The reaction site of rotenone and of barbiturates does not appear to be the same, however, in the soluble flavoprotein extracted by heat-acid-alcohol as in intact particles, but may be a new site created or revealed by the modifying effects of the extraction method (Horgan and Singer, 1967).

Since DPNH-cytochrome c reductase and DPNH-CoQ reductase are extracted under very similar conditions and since heat-acid-ethanol are responsible for the emergence of both activities, it was of interest to examine the relation of DPNH-CoQ reductase to the cytochrome c reductase of Mahler et al. (1952). The two preparations have already been compared with respect to composition, catalytic activities, and chromatographic behavior and no significant difference was found (Machinist and Singer, 1965 a,b). On the other hand Pharo et al. (1966) and Sanadi et al. (1965) have emphasized that, despite the similarity of the two preparations, only DPNH-CoQ reductase can react with long chain CoQ homologues, while the Mahler enzyme cannot. The recent publication of the assay method used by these workers (Pharo et al., 1966) has enabled us to

*Supported in part by grants from the National Science Foundation (GB 5352), the National Heart Institute (HE 10027) and by contract Nonr 5024 (00) between the Office of Naval Research and the University of California.
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examine this purported difference between the two flavoproteins.

DPNH-cytochrome c reductase was prepared from "pH 5.4 particles" derived from heart muscle by the original procedure of Mahler et al. (1952) except for one modification: lyophilization of the acid-ethanol extract was omitted since it has been found that this treatment is destructive to CoQ reductase activity (Salach et al., 1967). In order to concentrate the dilute alcohol extract, the enzyme was adsorbed on calcium phosphate gel (5 to 6 mg./mg. protein) and eluted with 5 ml. K_2HPO_4, pH 8.0, per 100 ml. alcohol extract. After desalting on Sephadex G-25, the enzyme was fractionated by the method of Mahler et al. Table 1 shows that the acid-ethanol extract contains rather high CoQ_6 reductase activity, which accompanies cytochrome c reductase activity during purification. The change in cyt. c/Q_6 activity ratio between R_3 and R_7 is not due to separation of the two activities but to the somewhat greater lability of Q_6 than of cytochrome c reductase activity, since none of the discard fractions contained appreciable DPNH-CoQ_6 activity and since in some other preparations the ratio remained nearly constant throughout the procedure.

Since the reductase is not homogeneous at the R_7 stage, it was further purified by chromatography on hydroxylapatite (Watari et al., 1963). The two activities are clearly associated with a single chromatographic component (Fig. 1). Examination of the eluted protein in the analytical ultracentrifuge showed it to be monodisperse.

DPNH-cytochrome c reductase extracted from ETP is in every known respect identical with the material from pH 5.4 particle (Watari et al., 1963). Several preparations from ETP have been studied with regard to inhibition of CoQ_6 and CoQ_{10} reductase activities by amytal and rotenone. DPNH-cytochrome c reductase was inhibited to the same extent and gave the characteristic rotenone titration curves as the authentic DPNH-CoQ reductase from phosphorylating particles (Salach et al., 1967).

Since neither reactivity with CoQ homologues, nor sensitivity to amytal or rotenone, nor any of the parameters previously examined revealed a clear-cut difference between the preparations of Mahler et al. (1952) and of Pharo et al. (1966), it was decided to purify the CoQ reductase from ETP_H in order to permit closer study of the two preparations as proteins. The flavoprotein was extracted as per Pharo et al. (1966), concentrated on calcium phosphate gel and fractionated to the R_3 stage with $(NH_4)_2SO_4$ as in Table 1. The specific activities of the initial extract were: cyt. c = 150, CoQ_6 = 20; cyt. c/CoQ_6= 7.5. At the R_3 stage the respective values were 282 and 34; the cyt. c/CoQ_6 ratio was 8.3. Fig. 2 shows the chromatographic pattern of the resulting material on hydroxylapatite. Only a minor fraction of the activity was eluted with 0.04 M

TABLE 1. PURIFICATION OF DPNH-CYTOCHROME c REDUCTASE

Purification Step	DPNH-CoQ_6 Activity		DPNH - Cyt. c Activity		Ratio: Cyt. c./CoQ_6
	Specific Activity	Total Activity	Specific Activity	Total Activity	
Alcohol extract	5.53	5,640	26.3	26,800	4.7
Calcium phosphate eluate	14.2	5,400	58.7	22,300	4.2
R_3	13.0	2,640	60.5	12,240	4.6
R_7	16.5	875	123	6,540	7.5

The enzyme was isolated from "pH 5.4 particles" (154 g. protein) without interruption. The hydroxylapatite step was performed immediately after the isolation of R_7. Total activity = µmoles DPNH oxidized/min.; specific activity = µmoles DPNH/min./mg. protein. CoQ_6 reduction was assayed as per Pharo et al. (1966) and cytochrome c reduction (V_{max}) as described by Watari et al.(1963). The loss between the gel eluate and the R_3 stages was much less extensive in other preparations.

phosphate; another small peak was eluted with 0.08 M phosphate, while the majority of the activity appeared in the 0.16 M phosphate eluate.

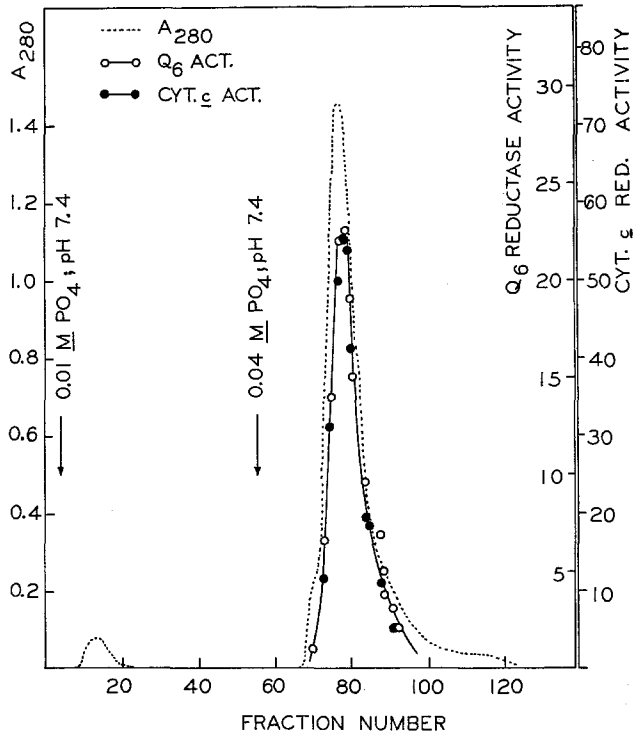

Fig. 1. Chromatography of DPNH-cytochrome c reductase on hydroxylapatite. The enzyme (42.5 mg. protein, R_7 stage, Table 1) was chromatographed in the dark at 0° on a 3.5 x 3.0 cm. column. Fractions of 2 ml. were collected at a flow rate of 40 ml./hr. The cytochrome c assays were at fixed acceptor concentration.

Sanadi (1966) has reported for his preparation, examined by the hydroxylapatite procedure of Watari et al. (1963), elution of the activity in two peaks (0.04 M and 0.08 M phosphate, respectively). Although the reasons for the discrepancy in observations between the two laboratories cannot be evaluated, since experimental conditions used by Sanadi have not been reported, the difference in chromatographic behavior of DPNH-CoQ reductase (Fig. 2) and DPNH-cytochrome c reductase (Fig. 1) are real and reproducible. Cochromatography of mixtures of the two preparations on hydroxylapatite gave the distribution expected from the data of Figs. 1 and 2.

The flavoprotein eluted with 0.16 M phosphate, pH 7.4 (Fig. 2) was usually found to be of the order of 90% homogeneous in the analytical ultracentrifuge. Cocentrifugation of CoQ and cytochrome c reductases in the double sector cell of the Spinco Model E showed identical sedimentation velocities ($s_{20,w}$ = 4.99 S

for the former and 4.96 S for the latter). FMN analysis of one sample of DPNH-CoQ reductase gave 1 mole flavin/74 x 10^3 g. protein. Thus the report (Pharo and Sanadi, 1964) that the CoQ reductase has a much higher molecular weight (180 x 10^3, based on FMN content) than the Mahler enzyme (accepted value = ca. 80 x 10^3) could not be confirmed.

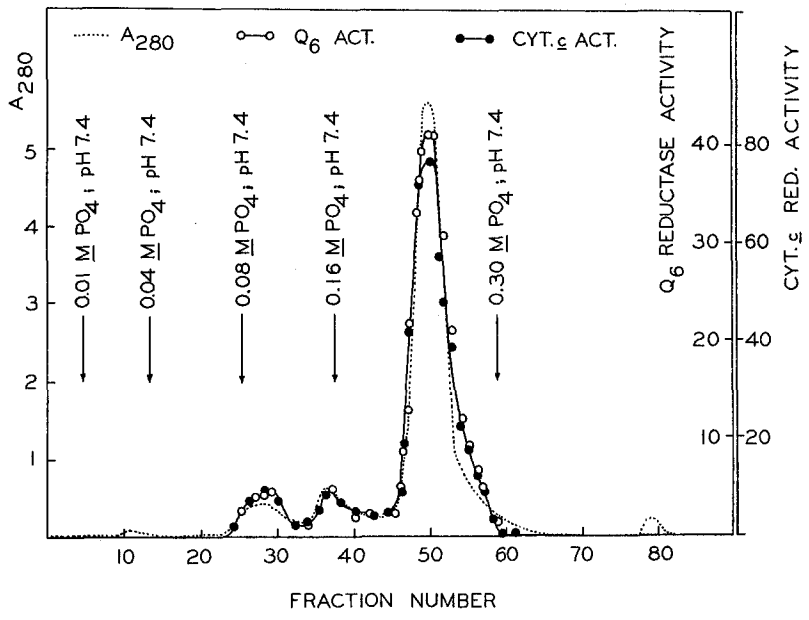

Fig. 2. Chromatography of DPNH-CoQ reductase on hydroxylapatite. The enzyme (R_3 stage, 95 mg. protein) was chromatographed on a 3.5 x 3.3 cm. column as in Fig. 1 and 3.3 ml fractions were collected and assayed immediately. The cytochrome reductase assays are at fixed acceptor concentration.

The two reductase preparations, therefore, show no meaningful differences in reactivity with ferricyanide, cytochrome c, menadione, CoQ_1, or long chain CoQ homologues, in molecular weight, spectrum, lability, and FMN, labile S, and nonheme iron content and both are inhibited partially by rotenone and amytal in the CoQ_6 reductase assay. There is a significant difference between the two, however, in behavior on hydroxylapatite columns. The only other difference which has been detected is a somewhat faster migration of the Mahler enzyme in analytical electrophoresis on polyacrylamide (0.03 M Tris - 0.02 M NaCl buffer, pH 9.0 at 0°, 27 V. cm^{-1}.) The simplest interpretation of these data is that both flavoproteins are fragments of the DPNH dehydrogenase molecule which arise as a result of conformation changes elicited by heat-acid-ethanol (Watari et al., 1963). The two reductases are very much alike in most of their properties; the differences in physical properties which have been noted may only reflect a minor difference in charge or confor-

mation. The fact that several components endowed with cytochrome c and CoQ reductase activity have been detected on hydroxylapatite (Fig. 2) after heat-acid-ethanol treatment, as after thermal, tryptic, or substrate-induced fragmentations (Cremona et al., 1963; Rossi et al., 1965) supports our view that the 80,000 molecular weight class reductases may be regarded as products arising from the breakdown of the tertiary and secondary structure of the dehydrogenase, whose structure depends on the particular conditions of fragmentation, rather than as a subunit of the enzyme.

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