Selective Extraction of DT Diaphorase from Mitochondria and Microsomes

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We recently reported the purification from the soluble fraction of rat liver cytoplasm of a diaphorase oxidizing DPNH and TPNH at equal rates, and reacting with a number of dyestuffs and quinones as electron acceptors [1]. The enzyme, called DT diaphorase, was characterized by a requirement for an activator, in the form of a dispersing agent such as serum-albumin or Tween-20, to exhibit maximal activity, as well as by a high sensitivity to dicoumarol. Although the bulk of the enzyme was found in the soluble fraction of the cytoplasm, portions of the enzyme were consistently recovered in the mitochondrial and microsomal pellets, and could not be removed from these by repeated washing with 0.25 M sucrose. However, when these pellets were suspended in 0.003 M phosphate-buffer, pH 7.5, and the suspensions treated in the cold with a Super-Thurrax blendor for 2 min, the clear supernatant obtained upon recentrifugation of the suspensions at high speed contained DT diaphorase. Moreover, the solutions so obtained were practically free from other diaphorase elements present in the mitochondria and microsomes. Illustrative data are summarized in Tables 1 and 2.

Table 1 shows an experiment with mitochondria. The blendorizing procedure employed here was adapted from that previously used by Kielley and Kielley [2] for the preparation of mitochondrial adenosine triphosphatase. It consisted of a treatment of the mitochondrial suspension with the Super-Thurrax blendor as described above, followed by a centrifugation of the treated suspension at 20 000 *g* for 10 min. The sediment was resuspended in 0.003 M phosphate buffer and the suspension was blendorized once again and recentrifuged as above. The two supernatants were combined and centrifuged at 105 000 *g* for 30 min. Three fractions were thus obtained: a "low-speed pellet", a "high-speed pellet", and a final supernatant. As shown in Table 1, only the final supernatant exhibited an appreciable TPNH diaphorase activity, and this was accompanied by an equally high DPNH diaphorase activity. Moreover, these activities were enhanced by 0.3 % Tween-20 and strongly inhibited by 10^{-6} M dicoumarol. By contrast, the DPNH diaphorase activities found in the low- and high-speed pellets were not stimulated (and even slightly inhibited) by 0.3 % Tween-20, and were completely insensitive to 10^{-6} M dicoumarol. When the supernatant solution was subjected to chromatography on DEAE-cellulose column, the TPNH and

Table 1. Diaphorase activities of mitochondrial subfractions. For fractionation procedure, see text. The test system contained: 0.04 mM 2,6-dichlorophenolindophenol (DCPIP), 0.1 mM DPNH or TPNH, 0.33 mM KCN and 0.05 M potassium phosphate, pH 7.5.

Fraction	Substrate	Diaphorase activity (μmoles DCPIP reduced / min / g liver) no add.	0.3 % Tween-20	10^{-6} M dicoumarol
Mitochondria	DPNH	3.93	3.93	3.57
	TPNH	0.71	1.61	0.36
Final Supernatant	DPNH	0.57	1.18	0.20
	TPNH	0.70	1.25	0.11
High-speed Pellet	DPNH	0.86	0.73	0.86
	TPNH	0.03	—	—
Low-speed Pellet	DPNH	1.34	1.01	1.34
	TPNH	0.04	—	—
Recovery	DPNH	2.77		
	TPNH	0.77		

Table 2. Diaphorase activities of microsomal subfractions. For fractionation procedure, see text. Test system as in Table 1.

Fraction	Substrate	Diaphorase activity μmoles DCPIP reduced / min / g liver: no add.	0.3 % Tween-20	10^{-6} M dicoumarol
Micro-somes	DPNH	3.31	1.95	3.40
	TPNH	0.37	0.70	0.25
1st Supernatant	DPNH	0.17	0.32	0.06
	TPNH	0.15	0.40	0.02
2nd Supernatant	DPNH	0.07		
	TPNH	0.03		
3rd Supernatant	DPNH	0.06		
	TPNH	0.01		
Final Pellet	DPNH	1.03	0.66	1.02
	TPNH	0.15	0.27	0.16
Recovery	DPNH	1.33		
	TPNH	0.34		

DPNH diaphorase activities were recovered in the same fraction.

An experiment with microsomes is shown in Table 2. Here, the treatment with the Super-Thurrax blendor was followed directly by centrifugation at 105 000 *g* for 60 min, after which the pellet was resuspended in 0.003 M phosphate-buffer, retreated with the blendor, and recentrifuged. The procedure was repeated three times altogether, thus resulting in three supernatant fractions and one final pellet. As seen in Table 2, the first supernatant exhibited equally high DPNH and TPNH diaphorase activities, and these were stimulated by 0.3 % Tween-20 and strongly inhibited by 10^{-6} M dicoumarol. The second and third supernatants exhibited only low TPNH diaphorase activities, and definitely higher, though still low, DPNH diaphorase activities. The final pellet revealed a high DPNH diaphorase activity, which was partially inhibited by 0.3 % Tween-20, and completely insensitive to 10^{-6} M dicoumarol. This fraction contained furthermore a definite portion, about half, of the TPNH diaphorase activity of the original microsomes. This TPNH diaphorase was, like the one found in the first supernatant, markedly enhanced by 0.3 % Tween-20, but in contrast, it was completely insensitive to 10^{-6} M dicoumarol.

The data presented above are consistent with the conclusion that DT diaphorase occurs both in mitochondria and microsomes, and can be selectively released from these elements after disruption of their structure by suitable means. Mitochondria seem to contain only one major enzyme with TPNH diaphorase activity and this is entirely accounted for by DT diaphorase. This conclusion is consistent with the previous finding of Stein and Kaplan [3] that column-chromatography of extracts of sonicated mitochondria yielded only one fraction with TPNH diaphorase activity, and that this fraction coincided with one of the two DPNH diaphorase fractions found by these authors. Microsomes, on the other hand, seem according to the present data to contain two types of TPNH diaphorase activity. One of these is accounted for by DT diaphorase, whereas the other seems to be independent of this enzyme as indicated by its resistance to dicoumarol and its firm association with the microsomal structure. The possible relation between this enzyme and the TPNH-neotetrazolium reductase recently found in microsomes by Williams *et al.*[4] is presently being investigated.

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