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Reconstitution of Apo-DT-diaphorase with Flavin-Adenine Dinucleotide

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The binding of flavin-adenine dinucleotide (FAD) in DT-diaphorase was studied. Incubation with 4 M guanidine–HCl at 0 °C rapidly dissociated FAD from the enzyme, and incubation of the apo-enzyme with FAD at 37 °C restored maximally about 50% (for menadione-reducing activity), or 330% (for ferricyanide-reducing activity) of the initial activity. The half-time required to reconstitute the apo-enzyme with FAD was 2 min for menadione-reducing activity and the K_m value of the apo-enzyme for FAD was 0.23 μM. These results show that DT-diaphorase contains non-covalently bound FAD as a prosthetic group.

Keywords——DT-diaphorase; reconstitution; flavin-adenine dinucleotide; non-covalent binding

Cytosol from various sources has a reducing activity on quinones. Most of this activity is catalyzed by a flavoprotein, DT-diaphorase, which is inhibited by the anticoagulant, dicoumarol.[1] The physiological function of DT-diaphorase seems to be a protective one against semiquinone radicals, which are readily autooxidized to produce the parent quinones and cytotoxic superoxide anion radicals,[2] because this enzyme catalyzes direct reduction of quinones to hydroquinones, not to semiquinones. DT-diaphorase has a dimeric structure of 51 kilodalton and contains flavin-adenine dinucleotide (FAD) as a prosthetic group.[3-5] However, removal of FAD and reconstitution with FAD have apparently not been done. Rase *et al.*[3] and Wallin[6] suggested the covalent binding of FAD with the polypeptide.

We characterized and purified DT-diaphorases from the livers of untreated, 3-methylcholanthrene-treated and 3, 4, 5, 3′,4′-pentachlorobiphenyl-treated rats.[7] During the course of this study, we found that the enzyme activity inactivated by guanidine–HCl treatment was restored by incubation with FAD at 37 °C. We report here data on the binding state of FAD in DT-diaphorase.

Experimental

Materials——Nicotineamide adenine dinucleotide (NADH), cytochrome c (horse heart, type III), FAD, flavin mononucleotide (FMN) and riboflavin were purchased from Sigma Chemical Company (St. Louis, MO); Sephadex G-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Purified DT-diaphorase was obtained as reported elsewhere.[7]

Enzyme Assays——DT-diaphorase activity was determined spectrophotometrically by following the increase in absorbance at 550 nm due to reduction of cytochrome c by menadiol formed at 37 °C. The assay mixture contained 40 μM menadione, 77 μM cytochrome c, 0.4 mM NADH and 50 mM Tris–HCl (pH 7.5) to make a final volume of 1 ml.

Preparation of Apo-enzyme——Purified DT-diaphorase was incubated with 4 M guanidine–HCl/0.1 M Tris–HCl (pH 7.5) for 1 min at 4 °C, and then applied to a Sephadex G-25 column (1.8 × 40 cm) equilibrated with 1% Tween 20/5% glycerol/50 mM Tris–HCl (pH 7.5). The void volume fraction was used as the apo-enzyme.

Results and Discussion

The purified DT-diaphorase, obtained as described elsewhere,[7] was rapidly inactivated by incubation with 2 and 4 M guanidine–HCl/0.1 mM ethylenediaminetetraacetic acid (EDTA)/0.1 M Tris–HCl (pH 7.5) at 0 °C (Fig. 1). The activity was decreased only slightly during the incubation with 2 or 3.6 M KBr, or 1 M guanidine–HCl. We attempted to restore the reduction activity of the 4 M guanidine–HCl-treated enzyme by 350-fold dilution with 0.1% Tween 20/71 mM Tris–HCl (pH 7.5) followed by incubation at 37 °C, but no activity was seen (Fig. 2). Restoration of activity was observed only when the guanidine–HCl-treated enzyme was incubated with FAD. The addition of FAD did not activate the untreated enzyme (data not shown). These data show that FAD is dissociated from the enzyme by 4 M guanidine–HCl treatment. The menadione-reducing activity could be recovered maximally to about 50% by incubation with 350 volumes of 10 μM FAD solution containing 0.1% Tween 20/71 mM Tris–HCl (pH 7.5) at 37 °C. Tween 20 was required to obtain a higher restoration rate. In these cases, the incubation time required for recovery of half-maximal activity was 2 min.

The inability to show complete reconstitution is presumably due to some irreversible denaturation. However, we cannot neglect the possibility that FAD might not be a proper prosthetic group, although this seems unlikely, because both the absorption and fluorescence spectra were the same as those of FAD (data not shown).

DT-diaphorase catalyzes a typical two electron reduction of quinones without forming semiquinones. It also catalyzes the reduction of ferricyanide, but not cytochrome c, both of which are one electron acceptors. When we examined the restoration of the ferricyanide-reducing activity, the activity reached maximally 330% of the control and the incubation time required to reach half-maximal activity was about 1 min (Fig. 2).

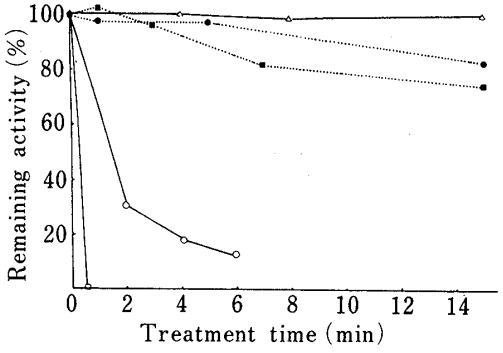

Fig. 1. Effect of Treatment with KBr and Guanidine–HCl on DT-Diaphorase Activity

Purified DT-diaphorase was incubated with 0.1 mM EDTA/0.1 M Tris–HCl (pH 7.5) containing 2 M KBr (△—△), 3.6 M KBr (●---●), 1 M guanidine–HCl (■---■), 2 M guanidine–HCl (○—○), 4 M guanidine–HCl (□—□) and was assayed as described under Experimental.

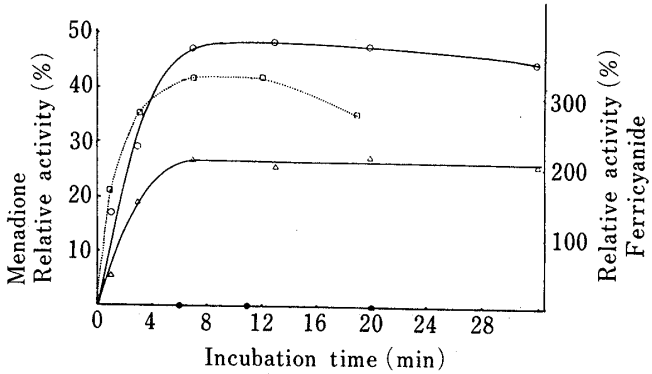

Fig. 2. Effect of Incubation at 37 °C with FAD and Tween 20 on the Activity of DT-Diaphorase Treated with Guanidine–HCl

DT-diaphorase was pretreated at 0 °C for 1 min in 4 M guanidine–HCl/0.1 mM EDTA/0.1 M Tris–HCl (pH 7.5). The sample was diluted 350-fold with 71 mM Tris–HCl (pH 7.5) containing the additions described below, and incubated at 37 °C for the indicated time. The activity recovered was then assayed by the addition of 0.4 mM NADH including 40 μM menadione/77 μM cytochrome c (solid line) or 1 mM ferricyanide (dotted line).

○—○, □---□, 10 μM FAD/0.1% Tween 20; ●—●, 0.1% Tween 20; △—△, 10 μM FAD. In the presence of Tween 20 in the reconstitution buffer, the activity of untreated enzyme in assay mixture containing Tween 20 was expressed as 100%, and in the absence of Tween 20, the activity in assay mixture without Tween 20 was taken as 100%.

Table I. Effect of FAD, FMN and Riboflavin in Restoring the Reducing Activity of DT-Diaphorase

Flavin	μM	Restoration of activity (%)
FAD	0.1	4.8
	1.0	44.8
	10	53.8
	50	38.1
FMN	10	1.1
	50	1.5
Riboflavin	10	0.6
	50	1.7

DT-diaphorase treated with 4 M guanidine–HCl was diluted and incubated at 37 °C for 10 min in a mixture containing the indicated concentrations of flavins in the presence of 0.1% Tween 20.

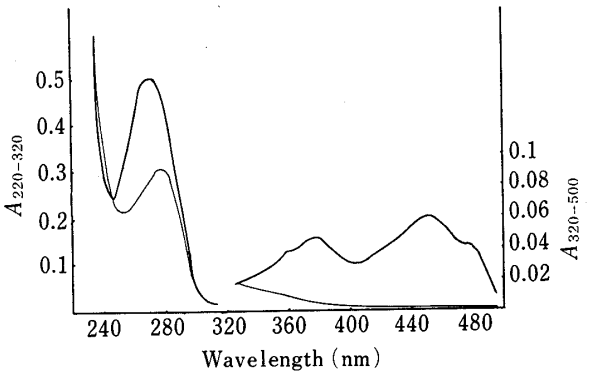

Fig. 3. Changes of Absorption Spectra of DT-Diaphorase on Guanidine Treatment Following Gel Filtration on Sephadex G-25

The holo-(——) and apo-(——) enzymes were dissolved at a concentration of 0.21 mg/ml in 1% Tween 20/5% glycerol/50 mM Tris–HCl (pH 7.5).

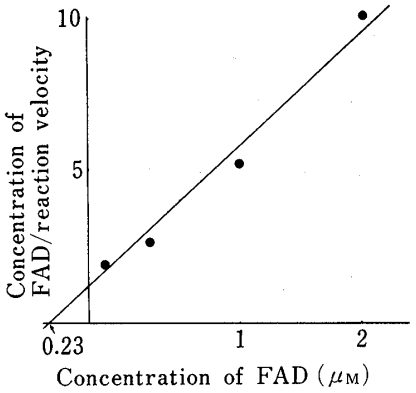

Fig. 4. Hanes–Woolf Plot of Reaction Velocity of the Reconstituted Enzyme *versus* Concentration of FAD Used

The apo-enzyme was incubated with the indicated concentration of FAD at 37 °C for 10 min and the activity was assayed. The K_m value was estimated to be 0.23 μM.

This reconstitution was specific for FAD, and neither FMN nor riboflavin showed any significant effect (Table I). The difference in the reducing activities of the reconstituted enzyme for menadione and ferricyanide (Fig. 2) may suggest that the reducing mechanism of one-electron acceptors such as ferricyanide differs from that of two-electron acceptors such as menadione. We observed that the menadione-reducing activity was increased 3.3-fold in the assay mixture containing 0.05% Tween 20, compared to that without the detergent, yet the ferricyanide-reducing activity was increased only 1.3-fold (data not shown). This supports the above proposal.

To confirm the dissociation of FAD from the enzyme, spectrophotometric examination was carried out before and after treatment with guanidine–HCl. Apo-enzyme was prepared by using Sephadex G-25 column chromatography in a solution buffered with 1% Tween 20/5% glycerol/50 mM Tris–HCl (pH 7.5) after guanidine–HCl treatment. The preparation aggregated in the absence of either Tween 20 or glycerol during the chromatography. Figure 3 shows the absorption spectra of the native enzyme and apo-DT-diaphorase. The native enzyme had absorption maxima at 271, 380 and 450 nm, and a characteristic shoulder at 480 nm, whereas in the apo-enzyme the typical peaks due to FAD at 380 and 450 nm had

disappeared and a peak at 271 nm was shifted to 278 nm, with decreased absorbancy. The apo-enzyme was unstable. Standing overnight at 4 °C and freezing–thawing resulted in complete inactivation. The K_m value of the apo-enzyme for FAD was estimated to be 0.23 μM from a Hanes–Woolf plot (Fig. 4).

In conclusion, it was clearly shown in the present study that DT-diaphorase contains noncovalently bound FAD as a prosthetic group.

References

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