32 PURIFICATION OF HEPATIC MICROSOMAL NADPH-CYTOCHROME P-450 REDUCTASE FROM LITTLE SKATE, RAJA ERINACEA

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The activity of cytochrome P-450-dependent monooxygenase systems in various species and tissues is dependent on several parameters, one of which is a supply of reducing equivalents. For the metabolism of many substrates NADPH-cytochrome P-450 reductase (cytochrome c reductase) (EC 1.6.2.4) is the source of electrons for the terminal oxidase. Thus, the study of this enzyme is essential to the elucidation of mechanisms of xenobiotic toxication and detoxication. The study of the skate reductase, besides applying specifically to the biochemical characterization of this marine species, may be important in determining the mechanism of reduction by the mammalian enzyme.

Preliminary studies (Serabjit-Singh, C.J. et al., Fed. Proc. 40:697, 1981) have revealed that in spite of significant differences between mammalian and marine reductases, the skate reductase is almost as active as the rabbit reductase in supporting metabolism mediated by rabbit hepatic cytochrome P-450, form 2, in a purified system. A rigorous comparison of these reductases is necessary to correlate the structure, redox potential and heat lability with enzymatic function. Therefore, a significant quantity of purified enzyme is required.

The yield of reductase from microsomes stored frozen for several months was quite variable. Often a low molecular weight form (monomeric molecular weight, 68,000 daltons, 68K reductase) which reduces cytochrome c but does not support cytochrome P-450 mediated reactions, was obtained. In an attempt to improve the yield of the fully active reductase (monomeric molecular weight, 74,000 daltons), freshly prepared (stored less than one week) microsomes from skate liver were used.

METHODS

Skate hepatic reductase was purified according to the affinity chromatography technique of Yasukochi and Masters (J. Biol. Chem. 251:5337, 1976) as modified by Serabjit-Singh et al., (J. Biol. Chem. 254:9901-9907, 1979) to separate the 68K reductase from the active enzyme. Phenylmethylsulfonyl fluoride ((PMSF), 0.25 mM, (Dignam, J.D., and Strobel, H.W., Biochem. 16:1116-1123, 1977) was added to the microsomes to inhibit proteolysis. The reductase was quantitated by its activity in reducing cytochrome c (1 unit = 1 nmol cytochrome c reduced/min/ml at 30°C) in 300 mM potassium phosphate buffer containing 3 mM magnesium chloride. Flavin content and composition was measured spectrally and fluorometrically (Faeder, E.T. and Siegel, L.M., Anal. Biochem. 53:332-336, 1974). Polyacrylamide gel electrophoresis was done according to the method of Laemmli (U.K.Nature (London) 227:680-685, 1970).

RESULTS

The yield of reductase (34%, Table 1) prior to chromatography on Sephacryl S-300 was similar to that obtained from rat or rabbit hepatic microsomes (data not shown). However, the final yield, 4% was 4 to 5 times lower than that from mammalian hepatic microsomes.

Two fractions were eluted from Sephacryl S-300, one in the void volume and the other was retained to the same extent as bovine serum albumin (molecular weight, 68,000 daltons). These fractions were concentrated by ultrafiltration, and the molecular weights, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, were identical, 68,000 daltons.

The ratio of flavin mononucleotide (FMN) to flavin adenine dinucleotide (FAD) per mole of reductase was 1. Subsequent to ultrafiltration, the content of total flavin was diminished 50%.

Table 1. -- Purification of little skate NADPH cytochrome c reductase

Preparation	Protein	Reductase		% Yield
		units/mg	units 10^3	
Microsomes	16.1 g[a]	104	1,682	(100)
DEAE Cellulose Eluate	———	—	1,159	68.9
2'5'-ADP Sepharose Eluate	———	—	575	34.2
Ultrafiltrate	———	—	445	26.5
Sephacryl S-300[b] Eluate	1.36 mg	54,800	74.1	4.4

[a]Six portions of 2-4 g were processed separately through step 3 after which the samples were pooled.

[b]Void volume fraction.

The 68K reductase exhibited a visible absorbance spectrum typical of a partially reduced flavin with an absorbance at 600 nm (Figure 1). The NADPH-reduced aerobic 68K reductase did not oxidize upon the addition of stoichiometric or excess amounts of patassium ferricyanide.

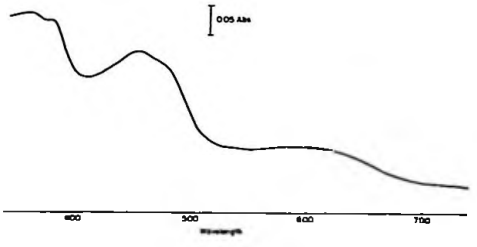

Figure 1.--Visible absorbance spectrum of NADPH cytochrome c reductase from liver of little skate, Raja erinacea.

DISCUSSION

The low yield of reductase and apparent degradation to the low molecular weight form may be due to either the intrinsic lability of the skate reductase as compared to the rat or rabbit flavoprotein, or due to proteases, not inhibited by PMSF, in skate hepatic microsomes.

The elution of the skate 68K reductase in the void volume of the Sephacryl S-300 column indicates that the marine 68K reductase aggregates, whereas the mammalian protein elutes as a monomer, suggesting that the skate 68K reductase may be more hydrophobic.

It would appear that freshly prepared skate hepatic microsomes offer no advantage over stored microsomes for the purification of the reductase. As the molecular weight of the reductase was not monitored during purification, the rate of formation of the 68K reductase is unknown. It is possible that during storage of the six 2'5'-ADP Sepharose column eluates (1 to 4 weeks) prior to final purification the degredation occurred, as it is at this step that the yield compares poorly with that of the mammalian reductase.

The spectral similarity between the 68K reductase and the 74,000 dalton reductase (Serabjit-Singh, C.J. et al., Fed. Proc. 40:697, 1981), suggests that the redox state of this protein may be independent of the hydro-

phobic region upon which support of cytochrome P-450 mediated metabolism depends (Dignam, J.D. and H.W. Strobel, Biochem 16:1116-1123, 1977). Thus, the 68K reductase should be useful in determining whether the e.s.r. detectable semiquinone is absent from the marine enzyme as indicated by our previous work. The authors wish to thank Steven Fisher and Chris D'Amico for their technical assistance.

33 VASOMOTOR RESPONSES OF SECRETORY AGENTS IN THE RECTAL GLAND OF SQUALUS - A PRELIMINARY STUDY

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In his studies on the physiology of the rectal gland, Burger (Physiol. Zool. 35:205-217, 1962) estimated blood flow to the gland from measurements of the chloride secretion rate and the arterialvenous difference in the concentration of this ion. Recalculations of the values he obtained show a very wide range of approximately 2-40 ml h^{-1} kg^{-1} (equivalent to some 80-2000 μl min^{-1} g $gland^{-1}$). In their study of the distribution of blood flow in Squalus, Kent et al., (Bull. MDIBL 13:64-66, 1973) obtained a mean value for the blood flow to the rectal gland of approximately 50 μl min^{-1} g^{-1} and it can be shown that this is in fact too low to sustain the known long-term secretory rates of sodium and chloride ions by the gland in vivo. The evidence suggests therefore that blood flow to the gland may be modulated during secretion and this has been confirmed by Solomon et al., (Bull. MDIBL 20:138-141, 1980), who showed that volume loading of the pithed fish stimulated rectal gland secretion and that this was accompanied by a significant increase in blood flow to the gland. Secretion in the isolated perfused gland can be stimulated by a variety of agents (e.g., cAMP, vasoactive intestinal peptide, adenosine) and the purpose of this investigation was to determine whether any of these agents produce vasomotor changes within the gland which might account for the observed in vivo changes in blood flow.

Glands were isolated and perfused essentially as described by Silva et al., (A.J.P. 233:F298-F306, 1977) except that the perfusion pressure was reduced to 20 mm Hg in order to more closely mimic in vivo dorsal aorta pressures (Satchell, Circulation in Fishes, C.U.P., 1971). Secretion rate, afferent (total) perfusion flow and efferent (via the large intestinal vein) perfusion flow were continuously monitored by means of rotameter-type flowmeters. Kent and Olsan (Bull. MDIBL 18:100-101, 1978) have shown that blood exiting via the large intestinal vein represents only that which has perfused the secretory parenchyma of the gland. Hence, by subtracting this efferent flow from the total afferent flow an estimate of the flow exiting from a variety of shunts bypassing the secretory cells can be obtained.

Initial experiments confirmed earlier reports on glands perfused at higher pressures (Silva et al., J. Memb. Biol. 53:215-221, 1980) that stimulation of secretion with various concentrations of vasoactive intestinal peptide (VIP) and cAMP is not accompanied by any changes in vascular resistance of the gland. At the pressure used in this study, efferent perfusion flow averaged some 1.72 ml min^{-1} g^{-1} and accounted for approximately 70% (55%-85%) of the total afferent flow. This flow rate is similar to the maximum value calculated from Burger's data and it therefore seemed possible that the unstimulated isolated gland, perfused at physiological pressures, was already in a highly vasodilated condition as compared to the situation in vivo.

In elasmobranchs, circulating levels of catecholamines are believed to play an important role in the maintenance of general vasomotor tone. In order to investigate the effects of catecholamines on vascular resistance in the isolated gland, different concentrations of various catecholamines were added to the perfusion fluid and the effect on perfusion flow was recorded. Typical results are shown in Figure 1 and indicate that low concentrations of norepinephrine produce a pronounced vasoconstriction within the isolated gland. Although complete dose-response curves have not been determined it is clear that with norepinephrine, significant vasoconstriction occurs over the concentration range of 10^{-8}M to 10^{-7}M. It is of some significance that this is within the normal