FLAVOCYTOCHROME b_2 OF BAKER'S YEAST : DISSOCIATION OF FLAVIN AND RECONSTITUTION OF LACTIC DEHYDROGENASE ACTIVITY.

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It has been shown by Appleby and Morton (1954) who first crystallized the L-lactic dehydrogenase of baker's yeast, that this enzyme has two prosthetic groups : one flavin (FMN) and one protohaem for a minimum molecular weight of about 80,000. The presence of FMN and haem was soon confirmed by Boeri et al. (1955). The exact importance of each of these groups in the enzymic transfer of electrons from lactate to the acceptors has not yet been clearly shown. Many observations led to the conclusion that dissociation of the flavin resulted in an inactive and unreactivable product.

Recently, in our laboratory, Iwatsubo succeeded in preparing a highly reactivable apoenzyme from the D-lactic dehydrogenase of anaerobic yeast (Iwatsubo and Curdel 1961). The flavin of this enzyme (FAD) is removed by an acid-ammonium sulfate precipitation method developped from that described by Warburg and Christian (1938). We have successfully applied the same method to the crystalline L-lactic dehydrogenase. We give here some results dealing with the preparation of the apoenzyme, specific reactivation by FMN, reactivation as a function of FMN concentration, and preliminary physico-chemical and kinetic properties of the apoenzyme and reconstituted flavoenzyme.

The haemoprotein apoenzyme is obtained from once crystallized flavocytochrome b_2, prepared according to Appleby and Morton (1959). Ammonium sulfate is added up to 35 % saturation to the solution of flavocytochrome b_2 (1 to 4 mg/ml). Temperature is kept at the limit of freezing during all subsequent treatments. The pH is brought to the range 2.2 to 1.8 with 2N HCl, then ammonium sulfate is added up to 70 % saturation and the precipitated protein is collected by centrifugation at -15°C. The treatment is achieved

in ten minutes or less. The precipitate is dissolved in phosphate buffer 0.1 M containing EDTA* 20 μM, at 0°C, and the clear solution adjusted to pH 7.2 with ammonia. There is rarely any insoluble material to be removed after dissolution. The FMN, measured in the yellowish supernatant from the absorption at 445 mμ agrees well with quantitative depletion.

Enzymic activities are measured in the Beckman DU spectrophotometer, at 30°C, in phosphate buffer pH 7.2, with EDTA 20 μM, DL-lactate 17 mM, and the acceptor (ferricyanide 0.66 mM or ferricytochrome c 50 μM). Owing to the effect of ionic strength on the reaction with ferricytochrome c (Boeri and Tosi 1956), buffer concentration was in this case 6.6 mM instead of 66 mM as was with ferricyanide.

The apoenzyme shows some residual lactic dehydrogenase activity with both ferricyanide and ferricytochrome c as acceptors. When FMN 10 μM is added in the test cells, the rate of reaction is immediatly increased to a level which is near 50 % of the original activity. Relative activities, expressed as percent of the original untreated enzyme, are given in the following table :

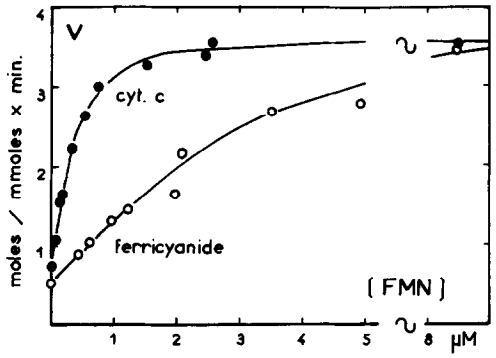
pH of acid A.S. precipitation	Residual activity added FMN = 0		Recovered activity by added FMN = 10 μM	
	Ferricyan.	Cyt. c	Ferricyan.	Cyt. c
2.0	6.6	9.0	40	43
1.95	6.0		43	
2.1	8.2	8.3	50	51
2.0	7.6	14	43	48

The reduction velocities for the two acceptors, expressed in moles of acceptor reduced per mole of apoenzyme and unit of time, are in the ratio 1:1 as for the native enzyme. No appreciable difference in the extent of inactivation or in the reactivation capacity was noted when acid treatments were made with either oxidized or lactate reduced enzyme. The apoenzyme can be kept during several hours at 0°C, or in the frozen state for some days without much loss of the reactivation capacity.

The apoenzyme is specifically reactivated by FMN. Riboflavin and FAD are both ineffective. Reactivation has been investigated as a function of FMN concentration, with either ferricyanide or ferricytochrome c as accep-

* EDTA = ethylene diamine tetra acetate.

tors, at 30°C and pH 7.2. Restitution of the ferricytochrome c activity occurs for concentrations of FMN lower than in the case of the ferricyanide activity. Experiments are being performed to explain this phenomenon.



Reactivation of the apoenzyme as a function of FMN concentration :

(pH = 7.2 and 30°C)

Apoenzyme : 60 mµM in the test with ferricyanide and 8 mµM in the test with ferricytochrome c.

Activities are expressed as moles of acceptor reduced per millimole of apoenzyme in one minute.

FMN is added in the test cells to make up the concentrations indicated.

The apparent Michaelis constants of the native and reconstituted enzyme for L-lactate are respectively : 1.8 and 6 mM with ferricyanide and 1.1 and 2.3 mM with ferricytochrome c, at pH 7.2 and 30°C.

When lactate is added to the oxidized haemoprotein, the spectrum of reduced haem appears immediatly. As noted by Morton (1961), the maxima correspond to the same wavelengths as for the reduced flavocytochrome b_2. Addition of dithionite gives only a small increase of the absorption. It is likely that the residual flavocytochrome b_2 acts as a mediator in the reduction of the haem of the apoenzyme. However, the possibility of a direct reduction is not entirely excluded.

It has been noted by several workers that the loss of activity of this enzyme which occurs in various circumstances is accompanied by an irreversible dissociation of flavin. Morton et al. (1959), suggesting that thiol groups were involved in the binding of flavin to the apoenzyme(hypothesis which received further experimental support from Armstrong et al.(1960)) stated that a reversible binding of FMN would require these groups to be in the reduced state. In fact, Boeri and Rippa (1961) have observed that the reversible part of the inactivation of the enzyme by urea was greater in the case of the lactate reduced enzyme. However, it is not certain that in this process the FMN is split from the apoprotein; and moreover, the haemoprotein isolated after urea treatment is not reactivable by FMN.

The data reported here show that a reversible binding of FMN to the haemoprotein is possible and the results are not affected by the oxidation state during the acid treatment. However the initial activity is not recovered to completion (50 %); this may be due to the denaturation of part of the apoprotein or to a lower maximal velocity for the reconstituted enzyme. In fact, several properties of the reconstituted enzyme differ from that of the native one. The apparent K_M for lactate are increased. Dilution does not inactivate the native flavocytochrome b_2 and addition of FMN never increased its activity, so that the flavin appears to be firmly bound to the protein. Results which are to be published shortly (Baudras 1962) show that dilution of the FMN-incubated apoenzyme result in a dissociation of the flavin. Investigations of the effect of pH on the activity with different acceptors (Baudras 1962) give results deviating from that obtained with the crystalline L-lactic dehydrogenase. It is probable that the bonds between FMN and the apoprotein are not integrally restituted. It is interesting to note that the enzymic activity does not require, here, an unambiguous binding of the FMN prosthetic group to the haemoprotein apoenzyme.

During the course of this work, Morton (1961) has reported the preparation of derivatives of the flavocytochrome b_2 containing only the flavin, or the haem, or none of these. These derivatives are devoid of lactic dehydrogenase activity, but some could be recovered after incubation of the haemoprotein with FMN, or of the apoprotein first with protohaemin then with FMN. The extents of reactivation of the haemoprotein as reported are : 12 % with ferricyanide and 1 % with ferricytochrome c as acceptors. The redissociation of flavin we have reported would perhaps account for these results, aliquots of the incubate being added to the test cells which contained no flavin.

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