XANTHINE OXIDASE, A MOLYBDO-FLAVOPROTEIN

by

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In 1939 we isolated a chromoprotein from milk which catalyzed the oxidation of hypoxanthine, aldehydes and DPNH[1]. Although FAD was identified as an essential part of the enzyme, the colour and absorption spectrum of the enzyme did not conform to that of typical flavoproteins. Concentrated solutions of the enzyme were deep red in colour. Furthermore only about 35% of the absorption at 450 mμ could be attributed to flavin. We were led to postulate the presence in the enzyme of a prosthetic group other than flavin which was responsible for the main part of the absorption in the visible spectrum. Other workers have confirmed these spectral anomalies in preparations of the milk enzyme isolated by various procedures[2-4].

Two recent observations have led us to reinvestigate the possibility of another functional group in the milk enzyme. DE RENZO *et al.*[5] have shown that the active constituent of a supplement which increases the level of xanthine oxidase in liver (WESTERFELD AND RICHERT[6]) was molybdenum. In our own laboratory MAHLER[7] has shown that butyryl CoA dehydrogenase is a cupro-flavoprotein which represents the first instance of a metallo-flavoprotein.

Xanthine oxidase was isolated from cream by a procedure similar to that previously described by us[1]. Analysis of the preparations at different stages of purity revealed the presence of molybdenum in a ratio of one atom of the metal per two molecules of flavin (cf. Table I). Samples were wet ashed, the residue was dissolved in hydrochloric acid and treated with potassium thiocyanate and stannous chloride. The molybdenum thiocyanate complex was extracted into ether and measured spectrophotometrically. This procedure is considered highly specific for molybdenum[8]. The absorption spectrum of the complex obtained from the ashed material was identical with that formed from a molybdenum standard with a maximum between 470 and 475 mμ. The presence of molybdenum in the enzyme was also confirmed by arc spectroscopy which showed the characteristic lines of this metal*. Corresponding blanks on the fluids of dialysis which was carried out before ashing were negative.

TABLE I

MOLYBDENUM AND FLAVIN CONTENT OF XANTHINE OXIDASE AT VARIOUS PURITY LEVELS

Preparation	*Flavin* micromoles/g	*Molybdenum* microgramatoms/g	*Flavin/Molybdenum* micromoles/microgramatom
A	0.74	0.35	2.1
A_1*	0.74	0.42	1.8
A_2**	1.54	0.72	2.1
B	1.76	0.88	2.0
B_1***	2.28	1.13	2.0

* A treated with cyanide
** Alcohol fraction of A
*** Alumina Cγ eluate of B

Flavin was determined spectrophotometrically in a 4% trichloroacetic acid extract of the enzyme. The absorption coefficient of FAD at 450 mμ was taken to be $9.83 \cdot 10^3$ $cm^2 \cdot mole^{-1}$ at the acid pH[9].

When the enzyme was coagulated by heat or exposed to 5% trichloroacetic acid, the metal was released into solution to the extent of 75% or more. However, no release was observed after exposure of the enzyme to 0.1 *M* cyanide at neutral pH.

* This analysis was kindly carried out for us by Professor V. W. MELOCHE of the Department of Chemistry, University of Wisconsin. The characteristic lines for Mo were observed at 3170.3, 3194.0, 3158.2, 3208.8 and 3233.1 Angström.

On the basis of PHILPOT's[10] calculation of the molecular weight and flavin content of xanthine oxidase it would appear that there are two molecules of FAD and one atom of molybdenum per molecule of enzyme with a molecular weight of about 230,000.

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Received August 10th, 1953

SYNTHESIS OF BUTYRYL-COENZYME A BY REVERSAL OF THE OXIDATIVE PATHWAY*

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The individual steps in the enzymatic oxidation of BuCoA*** and their reversibility have recently been established[1,2]. The present communication presents evidence for the overall synthesis of BuCoA from AcCoA in a system of six purified enzymes.

The essential sequence of reactions is: AcCoA $\overset{\text{I}}{\rightleftharpoons}$ AcAcCoA $\overset{\text{II, DPNH}}{\rightleftharpoons}$ β-OH-BuCoA $\overset{\text{III}}{\rightleftharpoons}$ crotonyl CoA $\overset{\text{IV, reduced benzylviologen}}{\rightleftharpoons}$ BuCoA, where I is the AcAcCoA cleavage enzyme[3,4,5], II is the β-OH-acyl CoA dehydrogenase[1,2,5,6], III is the unsaturated acyl CoA hydrase[1,2,5,7] and IV is the BuCoA dehydrogenase[1,8,9]. DPNH was generated from DPN by the oxidation of lactate catalyzed by lactic dehydrogenase (V)[10]. Benzylviologen was maintained in the reduced state by the oxidation of hypoxanthine catalyzed by xanthine oxidase (VI)[11].

The complete system consisted of $MgCl_2$, 2 μM; DPN, 3 μM; $CH_3{}^{14}COCoA$ (150,000 c.p.m. per μM) 2 μM; potassium lactate, 100 μM; benzylviologen, 0.5 mg; hypoxanthine, 1.5 mg; I, 0.04 mg; II and III, 0.2 mg; IV, 2.5 mg; V, 0.6 mg; VI, 2 mg; water to 1 ml. The reaction mixture was adjusted to pH 6† and incubated anaerobically for one hour at 38° C.

After deproteinization with perchloric acid, the CoA derivatives were extracted with phenol-benzyl alcohol[12] and the hydroxamic acids prepared by treatment with an excess of neutral hydroxyl-

* This work has been supported in part by contract AT 11-1-64 between the U.S. Atomic Energy Commission, Division of Biology and Medicine and the University of Wisconsin.
** Fellow, Heart Institute, National Institutes of Health. Additional support from the Helen Hay Whitney Foundation is gratefully acknowledged.
*** The following abbreviations are used: BuCoA (butyryl Coenzyme A thioester), Ac (acetyl), AcAc (acetoacetyl), DPNH (reduced diphosphopyridine nucleotide).
† A low pH was used to favor the reduction of AcAcCoA since this DPNH dependant reaction is markedly influenced by H^+.