AN ENZYME SYSTEM FOR CYCLIC KETONE LACTONIZATION[1]

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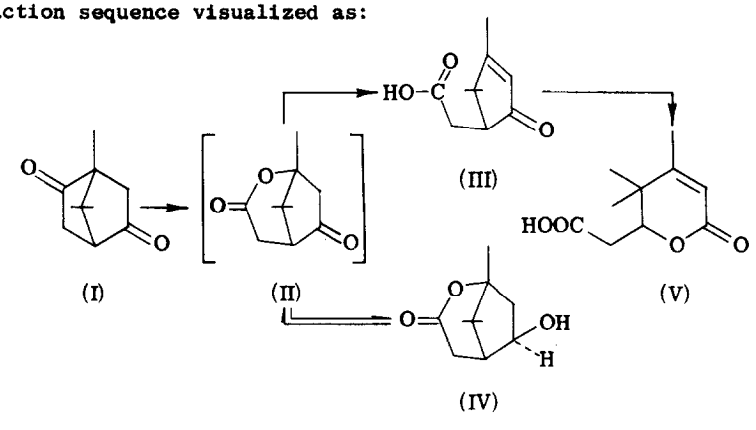
Camphor oxidation by a pseudomonad, strain C.1, proceeds via hydroxylation, dehydrogenation, and lactonization to the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid, V, an acyclic intermediate in the complete oxidation of (+)-camphor (Bradshaw, et.al., 1959). Both carbocyclic rings of camphor are cleaved by lactonization with the conversion of a cyclic ketone to the corresponding lactone (Conrad, et.al., 1961). This communication describes the enzymatic lactonization of 2,5-diketocamphane by enzymes obtained from the camphor-grown organism.

Cells were harvested from a 15-hour culture grown with aeration at 30°C in a mineral salts medium with (+)-camphor as carbon source and ground with glass beads in a micromill.[2] Centrifugation at 10^5 x g for two hours yielded a supernatant, which, when supplemented with DPNH in the presence of O_2, converts 2,5-diketocamphane (I) to 3,4,4-trimethyl-5-carboxy-methyl-Δ^2-cyclopentenone (III) in a reaction sequence visualized as:



(I) (II) (III) (IV) (V)

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[2] Gifford Wood Company, Hudson, New York.

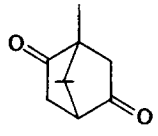
Unfractionated Spinco supernatants with sufficient DPNH added for conversion of I to III also catalyze the formation of IV (Hedegaard, et.al., 1961). Such extracts contain a DPN-linked dehydrogenase converting IV to III, again without accumulation of II. Thus II is implicated as an intermediate too unstable to be isolated under the experimental conditions. Purified enzyme preparations stoichiometrically convert I to III; extinction of the latter at 233 mμ (1.5×10^4 cm^2 $mmole^{-1}$) permits spectrophotometric assay.

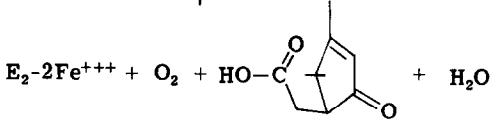
In addition to DPNH and O_2, the lactonization requires a dialyzable cofactor (FMN), ferrous iron and 2 enzyme fractions, E_1 (an FMN reductase) furnishing electrons, and E_2 (a lactonizing enzyme) which appears to undergo oxidation-reduction and to react with substrate and oxygen as follows:

(1) $DPNH + H^+ + FMN \xrightarrow{E_1} DPN^+ + FMNH_2$

(2) $FMNH_2 + E_2\text{-}2Fe^{+++} \longrightarrow FMN + E_2\text{-}2Fe^{++}$

[(2a) $FMNH_2 + O_2 \longrightarrow FMN + H_2O_2$]

(3) $E_2\text{-}2Fe^{++} + O_2 +$ 



The cofactor requirement is satisfied equally by boiled extract or FMN with maximal activity at 10^{-6}M; FAD shows one-third the activity of FMN and riboflavin is inactive. The E_1 dependent step (reaction 1) is not bipyridine sensitive, whereas the total reaction sequence 1 to 3 is (Table 1). Enzyme 1 can be estimated independently by O_2 uptake (side reaction 2a) or by DPNH oxidation (reactions 1 + 2a); both of these E_1 assays and the lactonization require FMN. Spinco supernatants made 10^{-3}M with respect to bipyridine and dialyzed vs several changes of buffer are completely inactive and can be reactivated by Fe^{++} reaching

TABLE 1

LACTONIZATION: ENZYME AND COFACTOR REQUIREMENTS

System	Amount	III Formed
	μmoles/ml	μmoles/hr.
1. Complete	---	0.96
2. -FMN	0.01	0.00
3. -G-6-P or -E_3[1]	10; 17U[1]	0.01
-DPN+	0.3	0.00
3a. -3+DPNH	3.0	0.32
4. -E_1[2]	15U	0.01
5. -E_2[3]	1U	0.00
6. 1 + bipyridine	1	0.00

[1] System (shaken in air 15' at 30°C) additions in μmoles/ml: 50 Tris, pH 7.2; 1.5 I; E_3 is Leuconostoc glucose-6-phosphate dehydrogenase=17 μmoles DPNH formed/hr.; + additions 2 to 5.
[2] E_1=(Fr.SAD) (Sp.A. 625); purified ca. 20 fold by $AmSO_4$ pptn. (0.35 to 0.5 Sat) and DEAE column. Assay -ΔO.D. at 340 mμ with FMN. Unit=1 μmole DPNH ox/hr.
[3] E_2= (Fr. SAH) (Sp.A. 40) purified ca. 20 fold by $AmSO_4$ pptn. (.5 to .65 Sat) and hydroxylapatite column. Assay + ΔO.D., 233 mμ; 1 μmole III = O.D. 15/ml. Unit=1 μmole III formed/hr.

maximal activity at 10^{-4}M; the following metals do not reactivate: Fe^{+++}, Co^{++}, Zn^{++}, Mn^{++}, Mg^{++}, Cu^{++}. Neither E_1 nor E_2 reacts with bipyridine (Table 2), but after reduction with the FMN reductase system (reactions 1 + 2), or chemically with $Na_2S_2O_4$, E_2 does react and reveals approximately 2 moles of ferrous iron (Table 2). Other reagents which reduce free ferric ions to the ferrous state, e.g., cysteine, glutathione, sodium borohydride and ascorbic acid, do not reduce the enzyme-bound iron and do not replace FMN in the lactonizing reaction. The figure of 2 moles reducible iron in the lactonizing enzyme, while compatible with first evidence of molecular weight and purity, is tentative pending more exhaustive evidence. Reactions 2 and 3 are suggested by these data. Coupling between reaction 1, catalyzed by E_1, and reaction 2, suggested as the first step with E_2, is not highly effective (Table 3) presumably because of the rapidity of side reaction 2a. The measurement of E_2 units is thus empirical.

The first enzyme, E_1, an electron transport oxidase in the terminology of Mason (1957), is visualized as serving the important function of returning the

TABLE 2

BIPYRIDINE REACTIVE IRON IN ENZYMES

Enzyme	E_1 (FMN Reductase)	E_2 (Lactonizing)
Enzyme:		
Specific Activity	2300	100
Fold Purified[1]	60	50
Protein, mg/ml	0.96	2.28
M x 10^{-5}	0.96[2]	2.28
Fe^{++} ion[3], M x 10^{-5}		
Before reduction	nil	nil
Reduced, $Na_2S_2O_4$	0.2	4.12
Reduced, $FMNH_2$[4]	---	4.05

[1] Calculated from centrifuged extract =1.
[2] Estimated molecular weight=10^5.
[3] Bipyridine added to $2x10^{-3}$M; Fe^{++}-bipyridine complex E_M^{520} = $1.43x10^4$ cm^2 $mmole^{-1}$.
[4] Reduced with complete system, Table 1, minus diketone substrate.

TABLE 3

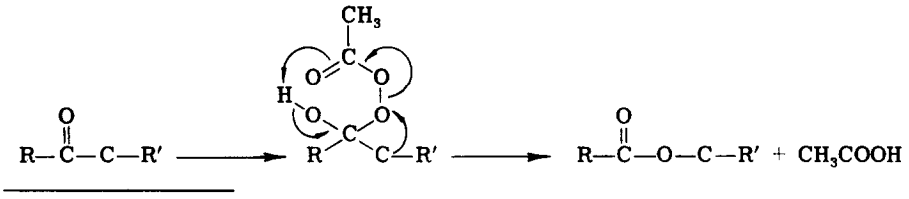
REDUCTASE-LACTONIZING ENZYME COUPLING

E_1[1]		E_2[2]	III Formed[3] Units E_2
µgProtein	Units	µgProtein	µmoles/hour
.0	.0	16.	0.0
5.	4.5	16.	.1
12.5	11.	16.	.38
25.	22.5	16.	.86
37.5	34.	16.	1.48
50.	45.	16.	1.86

[1] E_1 (FMN reductase) Sp.A. 900 $AmSO_4$ ppt. (.35-.5 Sat) + Hydroxylapatite and DEAE columns. 0.25 mg Protein ml (U=1 µmole DPNH oxidized/hr.).
[2] E_2 (lactonizing enzyme) $AmSO_4$ ppt. (.50-.65 Sat) + DEAE column. 0.78 mg protein ml.
[3] Assay contains/ml; complete system, Table 1, + enzymes as indicated.

lactonizing enzyme E_2 to an active "ferrous" state following oxidation during each substrate conversion. Reaction 2 appears to be quite specific for reduced flavin mononucleotide but may be non-enzymatic.

The lactonization is similar to several other enzyme-catalyzed reactions e.g., the lactic oxidative decarboxylase (Sutton, 1957), the cleavage of the C-17 side chain from progesterone and lactonization of the D ring of testosterone (Vischer and Wettstein, 1953), and the cleavage of inositol to glucuronic acid (Charalampous, 1960). These are all mixed function oxidases (Mason, 1957) with the electrons derived from various sources depending on the particular system. All may be considered together as a single reaction type; i.e. enzymatic cleavage of a carbon-carbon bond of an aliphatic or alicyclic ketone, presumably by a peroxidative attack at the carbonyl carbon followed by migration of an adjacent carbon to the oxygen of the added peroxide, thus forming the ester or lactone with release of the reduced peroxide. This mechanism has been proposed for the Baeyer-Villager conversion of ketones to esters or lactones by peracids. (Meinwald and Frauenglass, 1960).



In the enzymatic reactions the attacking peroxide(s) have not been identified. They appear to be formed by the reduction of oxygen by flavin or ferrous ions, or both, with substrate or reduced pyridine-nucleotide acting as the ultimate source of electrons.

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