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β-CYCLOPIAZONATE OXIDOCYCLASE FROM *PENICILLIUM CYCLOPIUM*

II STUDIES ON ELECTRON ACCEPTORS, INHIBITORS, ENZYME KINETICS, AMINO ACID COMPOSITION, FLAVIN PROSTHETIC GROUP AND OTHER PROPERTIES*

J C SCHABORT AND D J J POTGIETER

Department of Biochemistry, Rand Afrikaans University, Johannesburg National Nutrition Research Institute of the South African Council of Scientific and Industrial Research, Pretoria, and the Department of Biochemistry, University of Pretoria, Pretoria (South Africa)

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SUMMARY

1 2,6-Dichlorophenolindophenol, phenazine methosulphate and cytochrome *c* were effective electron acceptors in the dehydrogenation reaction catalysed by the β-cyclopiazonate oxidocyclases Oxygen was an effective terminal electron acceptor, but only in the presence of phenazine methosulphate as an intermediate electron carrier Other electron acceptors and carriers were studied as was the effect of various metal ions and possible inhibitors Most bivalent metal ions were found to inhibit the enzymes Inhibition of the dehydrogenation and cyclization reactions by 2,4-dinitro-1-fluorobenzene and L-1-tosylamide-2-phenylethylchloromethyl ketone indicates the possible roles of amino groups and the histidine imidazole group in these reactions Similar conclusions were made from studies of the effect of pH on the reactions Mechanisms involving the abovementioned groups are proposed for the dehydrogenation as well as the cyclization reaction

2 The thermodynamic properties of the isoenzymes were found to be similar With respect to the dehydrogenation reaction, the following values were found activation energy (E_a approx 7 2 kcal mole^{-1}), enthalpy change of activation (ΔH^* approx 6 6 kcal mole^{-1}), entropy change of activation (ΔS^* approx −42 cal mole^{-1} degree^{-1}), and free energy change of activation (ΔG^* approx 19 3 kcal mole^{-1}) The five isoenzymes were also similar with respect to their response towards inhibitors and electron acceptors, K_m values for 2,6-dichlorophenolindophenol (K_m approx 1 6 μM, isoenzyme II somewhat higher) and catalytic constants (k_2 approx 0 048 sec^{-1}, isoenzyme II somewhat lower)

3 The isoenzymes of β-cyclopiazonate oxidocyclase differed in the order of the dehydrogenation reaction with respect to β-cyclopiazonic acid, the half periods of the dehydrogenation reaction, the Michaelis constants for β-cyclopiazonic acid (2 1–14

Abbreviations DCIP, 2,6-dichlorophenolindophenol, PMS, phenazine methosulphate, TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone, DNFB, 2,6-dinitro-1-fluorobenzene, PCMB, *p*-chloromercuribenzoate

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μM), amino acid composition as far as the ratio of basic to acidic amino acids are concerned, and ammonia and carbohydrate content

4 All the isoenzymes contained one covalently linked molecule of flavin per molecule of enzyme Absorption spectra of the isoenzymes showed characteristic peaks at 276, 366 and 450 nm and a shoulder at 290 nm Their fluorescence emission spectra showed a peak at about 527 nm and the flavin residue (most probably FAD), released by proteolytic digestion with pronase showed a fluorescence peak at 518 nm similar to that of FMN and FAD

INTRODUCTION

The conversion of β-cyclopiazonic acid into α-cyclopiazonic acid has been found to be catalysed by the β-cyclopiazonate oxidocyclases, which consists of five isoenzymes[1] Reliable and accurate assay methods as well as the isolation, purification and some physical properties have been described for these isoenzymes[1] In order to obtain more information about the properties of the isoenzymes, kinetic and chemical studies and studies concerning other enzyme characteristics were performed

EXPERIMENTAL PROCEDURES

Materials

α-Cyclopiazonic acid was isolated from cultures of *Penicillium cyclopium* Westling (strain 1082) on sterilized wet maize meal as described by HOLZAPFEL[2] β-Cyclopiazonic acid was isolated from shake cultures of *P cyclopium* Westling (strain 1082) grown in aqueous media described elsewhere[3,4]

$NADP^+$, NAD^+, FAD and FMN were obtained from Boehringer, Mannheim, Germany Phenazine methosulphate (PMS), *p*-chloromercuribenzoate (PCMB), L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), antimycin A, 1,10-phenanthroline, trypsin, chymotrypsin and coenzyme Q6 were obtained from Sigma Chem , St Louis, Mo , U S A 2,6-Dichlorophenolindophenol (DCIP) was obtained from E Merck, Darmstadt, Germany, 2-methyl-1,4-naphthoquinone from Eastman Organic Chemicals, Rochester, N Y , U S A , methylene blue from Hopkin and Williams, Essex, England, 2,6-dinitro-1-fluorobenzene (DNFB) from British Drug Houses, Johannesburg, South Africa, and pronase from Calbiochem, Los Angeles, Calif , U S A All other chemicals used were of analytical grade quality

The β-cyclopiazonate oxidocyclases

The five isoenzymes of β-cyclopiazonate oxidocyclase were purified from the mycelium of *P cyclopium* Westling as described elsewhere[1]

Assay of β-cyclopiazonate oxidocyclase activity

The spectrophotometric assay methods using DCIP and cytochrome *c* as terminal electron acceptors were employed to determine the dehydrogenating activity of the isoenzymes in most of these studies A decrease of 0 001 in absorbance per min at 600 nm was used as an arbitrary unit of activity when employing DCIP as terminal electron acceptor Activity was normally expressed in nmoles DCIP or cytochromec

reduced or β-cyclopiazonic acid dehydrogenated per min and specific activity in nmoles/min per mg protein All determinations were made at 25° and pH 6 82 (except during pH studies) Protein concentration was determined by a standardized method of LOWRY *et al* [5] and total nitrogen by a micro-Kjeldahl method[6]

Carbohydrate determination

The carbohydrate content of the isoenzymes was determined by the anthrone method described by ROE AND DAILEY[7]

Pentose determination

The pentose content of purified enzyme preparations and the flavin residues of the isoenzymes after release from the protein by pronase digestion and separation on paper chromatograms was determined according to the method of MILITZER[8] as described by MARKHAM[9]

Determination of acid labile inorganic sulphur

The acid labile inorganic sulphur content of the five isoenzymes was determined by the method described by LOVENBERG *et al* [10]

Amino acid analyses

Amino acid analyses were performed in a Beckman Spinco Model 120-C amino acid analyzer Solutions of the five isoenzymes of β-cyclopiazonate oxidocyclase were dialysed exhaustively against deionized water and then lyophilized Samples of these enzymes (200–700 μg) were dissolved in 2 ml 6 M HCl in Pyrex tubes, frozen, evacuated and sealed under reduced pressure Hydrolysis was performed at 110 ± 2° for 24 h

Half-cystine was determined in samples of isoenzyme I and III as cysteic acid after performic acid oxidation under reflux for 24 h Tryptophan was determined by employing the spectrophotometric method of BENCZE AND SCHMID[11]

Studies on the flavin prosthetic group of the β-cyclopiazonate oxidocyclases

Spectrophotometric studies in the ultraviolet and visible regions were performed in a Beckman model DK2A ratio recording spectrophotometer at pH 6 7 in 0 02 M sodium phosphate buffer containing 0 08 M NaCl

Attempts to release the flavin from the enzyme by conventional methods such as treatment with cold acid[12,13] (6 and 10% (w/v) trichloroacetic acid) or by thermal denaturation[14] (boiling for 15 min) were unsuccessful Chymotrypsin and trypsin, used separately as well as combined, were also unable to release the flavin from the enzymes Proteolytic digestion with pronase, however, yielded better results Pronase digestion was carried out (0 5 mg of pronase per mg of enzyme protein) at pH 7 1 in 0 05 M Tris–HCl buffer for 4 h at 38° The isoenzymes were precipitated with 6% (w/v) trichloroacetic acid (final concentration) at 0° After centrifugation the precipitate was used for proteolysis by suspending it in the buffer and adjusting the pH to 7 1 by addition of Tris when necessary Undigested protein was coagulated by heating for 10 min at 95° and then removed by brief centrifugation The supernatant obtained contained the flavin

Paper chromatography of the flavin residues was performed essentially as described by KEARNEY[15]

Fluorescence spectra of the β-cyclopiazonate oxidocyclases as well as the flavin residues released from the proteins by pronase digestion were determined in 0 03 M Tris–HCl buffer (pH 7 1) in a Farrand spectrofluorometer employing a Xenon lamp power supply and filter No 373 This filter absorbed light at wavelengths below 420 nm

RESULTS AND DISCUSSION

Electron carriers, electron acceptors and cofactor requirements for the reactions catalysed by the β-cyclopiazonate oxidocyclases

Ferricyanide, 2-methyl-1,4-naphthoquinone, methylene blue, coenzyme Q6, NAD^+, $NADP^+$, FAD and FMN did not act as electron acceptors or carriers for the dehydrogenation of β-cyclopiazonic acid and also showed no effect on the formation of α-cyclopiazonic acid under aerobic as well as anaerobic conditions Where PMS showed the ability to increase the dehydrogenating activity (when DCIP or cytochrome *c* was used as terminal electron acceptor) as well as the rate of the conversion of β- into α-cyclopiazonic acid in the presence of DCIP, cytochrome or oxygen, compounds such as 2-methyl-1,4-naphthoquinone and coenzyme Q6 did not show similar abilities

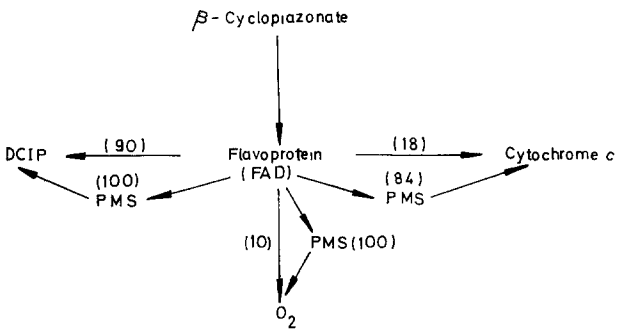


Fig 1 Proposed electron-transfer sequences associated with the dehydrogenating activity of the β-cyclopiazonate oxidocylases One molecule of flavin was found per molecule of enzyme The flavin moiety appeared to be FAD or a derivative of FAD The numbers in parentheses indicate activities relative to the activity of PMS + DCIP taken as 100%

Cyrochrome *c*, DCIP, PMS and oxygen have been described as terminal or intermediate electron acceptors in assay methods for many flavoprotein and metallo-flavoprotein enzymes Oxygen did not appear to be a very effective direct acceptor of electrons from the flavoproteins except in the presence of PMS, which probably acted as an intermediate electron carrier Results obtained indicated that PMS can accept electrons from the β-cyclopiazonate oxidocyclase flavoproteins at the same rate under aerobic and anaerobic conditions The presence of oxygen had no effect on the dehydrogenating activity of the enzymes when DCIP or cytochrome *c* were used as terminal electron acceptor A similar observation was made for succinic dehydrogenase[16]

From available results concerning electron carriers and acceptors, a scheme of

electron-transfer sequences associated with the dehydrogenating activity of the β-cyclopiazonate oxidocyclases is proposed in Fig 1

The highest reaction rate obtained for the dehydrogenation reaction, similar to that obtained for the conversion of β- into α-cyclopiazonic acid in the presence of PMS, was observed when a combination of PMS and DCIP was used The finding indicated that PMS seems to be the most effective immediate acceptor of electrons and that the dehydrogenating reaction may be the rate limiting reaction in the conversion of β- into α-cyclopiazonic acid In all reaction mixtures α-cyclopiazonic acid was formed at approximately the same rate as the rate of dehydrogenation, indicating that the cyclization reaction may be in concert with the dehydrogenation reaction The isoenzymes thus catalyze both reactions, first the dehydrogenation reaction followed by the cyclization reaction, while the substrate is most probably bound to one binding site on the enzyme molecule It can be expected that a compound similar in structure to the proposed intermediate will not be released after the dehydrogenation reaction This hypothesis may be supported by the fact that compounds containing an indole system conjugated to a double bond are unstable[2,4] and should polymerize when released from the active centre of the isoenzymes prior to the cyclization reaction The formation of α-cyclopiazonic acid from β-cyclopiazonic acid as catalysed by the β-cyclopiazonate oxidocyclases differs markedly from the conversion of squalene into lanosterol[17] in that the oxidation and cyclization reactions seem in concert and catalysed by one single enzyme molecule

The reverse reaction, *viz* the conversion of α- into β-cyclopiazonic acid was studied by a method based on that used by SINGER *et al* [18,19] for studying the reoxidation by fumarates of $NaHSO_3$-reduced succinic dehydrogenase Results indicated that the isoenzymes could not catalyse the reverse reaction under the experimental conditions used (pH 6 6) It may also be concluded that under the conditions of the culture medium (pH about 5 5) the conversion of β- into α-cyclopiazonic acid also appeared to be fairly irreversible

Inhibition studies

Two assay methods were used in inhibition studies, *viz* dehydrogenating activity employing DCIP as terminal electron acceptor and the total conversion of β- into α-cyclopiazonic acid by the Ehrlich method The results are presented in Table I Similar results were obtained for all five isoenzymes

All the divalent cations tested have been reported to be moderate to strong inhibitors of certain flavoprotein enzymes such as the NADH dehydrogenases[20,21] Mg^{2+} was found to be an activator of certain enzymes[20] The important role of trace elements like zinc and iron in the production of α-cyclopiazonic acid in the cultures of *P cyclopium* Westling is definitely not attributable to their action as cofactors or activators of β-cyclopiazonate oxidocyclase They may, however, have a direct or indirect effect on the synthesis of these isoenzymes Monovalent cations and anions such as phosphate, maleate, cyanide and sulphide had no effect on enzyme activity Sulphide interfered with the assay method employing DCIP 1,10-Phenanthroline appeared to have no effect on enzyme activity This may indicate the absence of metals in the enzyme molecule, although no inhibitory effects towards some metalloenzymes have been reported as yet The fact that citrate, phosphate, cyanide, sulphide and maleate did not show any inhibitory effect supports the conclusion that the

TABLE I

EFFECT OF INHIBITORS ON THE REACTIONS CATALYSED BY β-CYCLOPIAZONATE OXIDOCYCLASE, ISOENZYME III

Because Fe^{2+} appeared to reduce DCIP and Fe^{3+} formed an orange-red complex with DCIP and so interfered with the DCIP method, the PMS–cytochrome *c* monitored reaction was employed to determine the dehydrogenating activity in the case of 1 mM $FeCl_3$ and 1 mM $FeSO_4$. These salts caused, respectively, 100 and 75% inhibition of the abovementioned reaction.

Reagent	*Concn (M)*	*Percentage inhibition*	
		DCIP method	*Ehrlich method*
$HgCl_2$	$1.2 \cdot 10^{-2}$	100	–
	$1.2 \cdot 10^{-3}$	98	73
	$6.0 \cdot 10^{-4}$	61	–
	$1.0 \cdot 10^{-4}$	8	–
$MnCl_2$	$1.0 \cdot 10^{-3}$	80	62
	$1.0 \cdot 10^{-7}$	7	–
$CoCl_2$	$1.0 \cdot 10^{-3}$	80	73
	$1.0 \cdot 10^{-6}$	22	–
$CuCl_2$	$1.0 \cdot 10^{-3}$	93	83
	$1.0 \cdot 10^{-7}$	6	–
$ZnSO_4$	$1.0 \cdot 10^{-3}$	83	71
	$3.3 \cdot 10^{-4}$	40	–
	$1.0 \cdot 10^{-6}$	6	–
	$1.0 \cdot 10^{-7}$	5	–
Na_2S	$1.0 \cdot 10^{-3}$	–	0
$CaCl_2$	$1.0 \cdot 10^{-3}$	35	22
$MgCl_2$	$1.0 \cdot 10^{-3}$	(+)10	0
	$1.0 \cdot 10^{-7}$	(+) 5	–
NaCl	$5.0 \cdot 10^{-2}$	0	0
Phosphate	$5.0 \cdot 10^{-2}$	0	0
Maleate	$5.0 \cdot 10^{-2}$	0	0
KCN	$1.0 \cdot 10^{-3}$	0	0
1,10-Phenanthroline	$1.0 \cdot 10^{-2}$	0	0
Antimycin A	$1.0 \cdot 10^{-4}$	0	0
PCMB	$1.0 \cdot 10^{-4}$	0	0
DNFB	$3.0 \cdot 10^{-3}$	16	18
	$1.0 \cdot 10^{-3}$	7	11
TPCK	$1.0 \cdot 10^{-3}$	3	19
$FeCl_3$	$1.0 \cdot 10^{-3}$	–	64
$FeSO_4$	$1.0 \cdot 10^{-3}$	–	66

oxidocyclases do not contain any metal ion. The absence of absorption peaks in the 420 and 520 nm regions, which were interpreted by SINGER *et al.*[22,23] to be due to iron–protein bonds, also indicates that iron does not appear to be bound to the enzyme molecules. According to emission spectroscopy the isoenzymes did not appear to contain any significant quantities of metal ions. It would appear, therefore, that the β-cyclopiazonate oxidocyclases differ from certain flavoprotein and metalloflavoprotein enzymes[24,25] that catalyse oxidation–reduction reactions in that they do not

contain any metal ion In this respect the isoenzymes correspond to the majority of flavoproteins and the flavodoxins[12,26]

PCMB did not show an inhibitory effect Antimycin A showed no effect on the activity, which may also indicate the absence of non-heme iron in these enzymes

Both the dehydrogenation as well as the conversion of β- into α-cyclopiazonic acid were inhibited by DNFB TPCK, however, has a somewhat greater inhibitory effect on the total conversion of β- into α-cyclopiazonic acid than on the dehydrogenation reaction This indicates that the cyclization reaction was inhibited by this compound and that this reaction may be an enzyme-catalysed reaction which appears to take place in concert with the dehydrogenation reaction It is obvious that inhibition of the dehydrogenation reaction should always lead to inhibition of the total conversion of β- into α-cyclopiazonic acid and does not mean that the cyclization reaction should also be inhibited It can be expected that TPCK reacted with an imidazole group in the active centre, but a α-amino or ε-amino group of lysine may also be involved A positively charged group on the enzyme molecule, which may be an imidazole or α-amino group, is expected to catalyse the cyclization reaction

Enzyme kinetics

Essentially similar results with the isoenzymes were obtained from kinetic studies on the effect of pH on the dehydrogenating activity of the β-cyclopiazonate oxidocyclases Seeing that the pH optimum of the dehydrogenation reaction is not dependent on substrate concentration and that a pH optimum of 6 8 was obtained, even at high substrate concentrations, it may be concluded, according to the data given by LAIDLER[27], that neither an acidic nor a basic group suggested to be involved

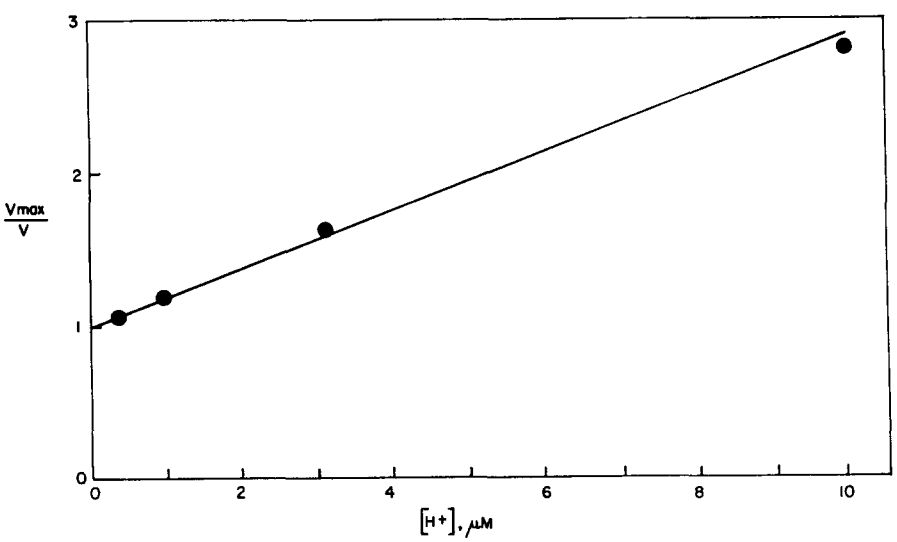


Fig 2 The determination of the dissociation constant of the basic group of isoenzyme III which is probably involved in the breakdown of the enzyme–substrate complex Enzyme activity or reaction velocity (v) was determined spectrophotometrically employing DCIP or PMS and cytochrome c as electron acceptors at different pH values v_{max} is the reaction velocity at the pH optimum

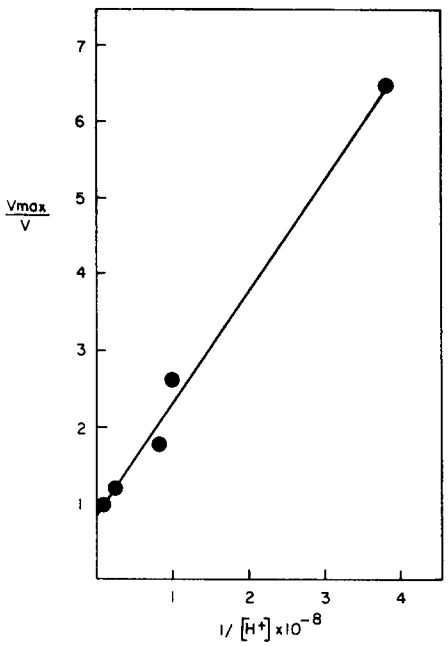
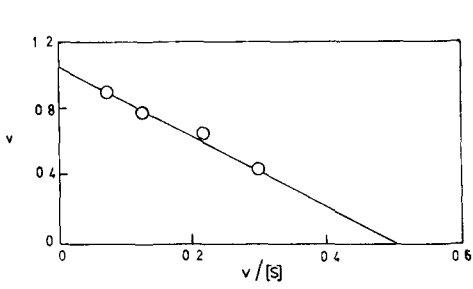


Fig 3 Determination of the dissociation constant of the acidic group of isoenzyme III which is probably involved in the breakdown of the enzyme–substrate complex Enzyme activity was determined as described in the legend to Fig 2

in this reaction is directly involved in the formation of the enzyme–substrate complex, but that the substrate may be bound to a neighbouring site Both groups, however, must play a part in the subsequent breakdown of the complex The $v_{max}/\bar{v}$ values, obtained at high substrate concentrations, were plotted against $[H^+]$ on the acid side of the pH optimum and against $1/[H^+]$ on the alkaline side (Figs 2 and 3) The v_{max} and $\bar{v}$ values were determined under zero order conditions (high [S] for DCIP and β-cyclopiazonic acid) The dissociation constants of the acidic (K_a) and basic (K_b) groups were calculated from the slopes[27]

According to LAIDLER[27] $K_a = K_a'$ and $K_b = K_b'$ in this case A pK_a' value of approx 7 8 and a pK_b' value of approx 5 3 were found The plots illustrated in Figs 2 and 3 are those obtained with isoenzyme III A plot of $\log_{10}\bar{v}$ against pH also yields pK_a' and pK_b' values of 7 8 and 5 3, respectively A pK_a value of 7 8 is consistent with the dissociation constant of an α-amino group[27] while a pK_b value of 5 3 falls nearly in the range of the ring nitrogen of the imidazole group of histidine[28] The possible role of an α-amino and an imidazole group in the dehydrogenation was also suggested by the inhibitory effects of DNFB and TPCK

The effect of substrate concentration on the dehydrogenating activity of the five isoenzymes was studied employing the spectrophotometric assay method with DCIP as terminal electron acceptor in the absence of PMS Apparent Michaelis constants for β-cyclopiazonic acid and DCIP were determined by the graphical methods of LINEWEAVER AND BURK[29] and EADIE[30] (examples of plots given in Figs 4 and 5) and are summarized in Table II



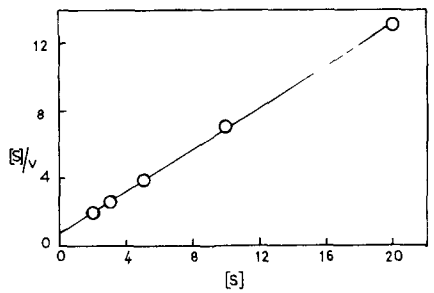


Fig 4 Eadie plot for the determination of the apparent K_m (β-cyclopiazonic acid) value of isoenzyme III of β-cyclopiazonate oxidocyclase The enzyme concentration used was 11 7 μg/ml ([S] is the concentration (μM) of β-cyclopiazonic acid and v is the reaction velocity of the dehydrogenation in nmoles/min)

Fig 5 Modified Lineweaver–Burk plot for determining the apparent K_m (DCIP) value of isoenzyme I of β-cyclopiazonate oxidocyclase The enzyme concentration used was 11 3 μg/ml ([S] is the concentration (μM) of DCIP and v the reaction velocity of the dehydrogenation reaction in nmoles/min)

K_m (β-cyclopiazonic acid) values varied considerably among the five isoenzymes while the K_m (DCIP) values showed less variation In comparison with the K_m values reported for the natural and artificial substrates of many other flavoprotein dehydrogenases[31–34] the values for β-cyclopiazonic acid and DCIP are lower

The differential method of LETORT[35] was used to determine the order (n) of the dehydrogenation reaction with respect to β-cyclopiazonic acid as well as the rate constants (K) under conditions where the reaction was zero order with respect to DCIP It was found that the half-period of the dehydrogenation reaction was dependent on the rate constant and the substrate and enzyme concentrations The half-periods (r) for the five isoenzymes given in Table III were determined at a β-cyclopiazonic acid concentration of 12 1 μM from the equation

$$r = \frac{2^{n-1} - 1}{(n-1)\, K_a{}^{n-1}} \quad \text{(see ref 35)}$$

The catalytic constants (k_2) for the five isoenzymes given in Table III were calculated from the relationship $v = K_2\,[E_0]$ at high substrate concentrations The molecular weight of the isoenzymes was taken as 50 000

TABLE II

K_m VALUES FOR β-CYCLOPIAZONIC ACID AND DCIP OBTAINED FROM LINEWEAVER–BURK AND EADIE PLOTS

	K_m for β-cyclopiazonic acid (μM)					*K_m for DCIP (μM)*				
Isoenzymes	*I*	*II*	*III*	*IV*	*V*	*I*	*II*	*III*	*IV*	*V*
Eadie plots	2 1	2 1	13 9	12 0	5 1	1 1	2 8	1 5	1 7	1 7
Lineweaver–Burk plots	2 4	2 2	14 8	11 7	5 1	1 0	2 8	1 4	1 4	1 4
Modified Lineweaver–Burk plots	1 9	2 4	14 4	11 2	4 9	1 1	2 8	1 3	1 8	1 8
Average	2 1	2 2	14 4	11 6	5 0	1 1	2 8	1 4	1 6	1 6

TABLE III

HALF-PERIODS, RATE CONSTANTS AND ORDER OF THE DEHYDROGENATION REACTION WITH RESPECT TO β-CYCLOPIAZONIC ACID, AS CATALYSED BY THE FIVE ISOENZYMES OF β-CYCLOPIAZONATE OXIDOCYCLASE

The values in parentheses indicate the enzyme concentrations in μg/ml employed in the determination of $t_{\frac{1}{2}}$

Isoenzyme	*Order with respect to β-cyclopiazonic acid*	*Rate constant (nmoles/sec)*	*Half-period ($t_{\frac{1}{2}}$) (min)*	*Catalytic constant (k_2) (sec^{-1})*
I	0.28	2.9	20 (11)	0.050
II	0.27	2.3	25 (9)	0.042
III	0.49	2.1	16 (12)	0.052
IV	0.51	1.2	28 (6)	0.048
V	0.25	1.6	37 (5)	0.047

Activation energies (E_a) were obtained for the dehydrogenation reaction during studies of the effect of temperature on enzyme activity from Arrhenius plots[35]. The entropy change of activation (ΔS^*), enthalpy change of activation (ΔH^*) and free energy change of activation (ΔG^*) were calculated by applying the theory of absolute reaction rates[35,36] and the following equations

$$\Delta H^* = E_a - RT$$

$$k_2 = \frac{kT}{h} e^{\Delta S/R} e^{-\Delta H^*/RT}$$

and

$$\Delta G^* = \Delta H^* - T\Delta S^*$$

The values of the thermodynamic quantities are given in Table IV

The relatively low values for the activation energy of the dehydrogenation reaction highlight the very efficient catalytic properties of these isoenzymes. The negative change in the entropy of activation, as also reported for most other enzyme-catalysed reactions, suggests a structural change (folding) in the enzyme molecules during formation of the activated complex. The possibility that the negative ΔS^* values may be attributed to an increase in polarity and a consequent binding of

TABLE IV

SOME THERMODYNAMIC CONSTANTS OF THE DEHYDROGENATION REACTION CATALYSED BY THE β-CYCLOPIAZONATE OXIDOCYCLASES

Isoenzyme	*Temp*	k_2 *(sec^{-1})*	E_a *(kcal/mole)*	ΔH^* *(kcal/mole)*	ΔS^* *(cal/mole per degree)*	ΔG^* *(kcal/mole)*
I	25.6°	0.050	6.75	6.15	−44.0	19.3
II	25.6°	0.042	8.36	7.77	−38.9	19.4
III	25.4°	0.052	8.26	7.67	−38.7	19.2
IV	25.5°	0.048	6.06	5.47	−46.4	19.3
V	25.2°	0.047	6.52	5.93	−44.9	19.3

water molecules during the formation of the activated complex seems unlikely, because the binding of a negatively charged substrate molecule to a positive group in or near the active site of the enzyme is a distinct possibility The positive change in the free energy of activation (ΔG^*) also falls within the range for many enzyme-catalysed reactions, especially the proteolytic enzymes, *i e* 13–19 kcal/mole[37] No general values have been reported for oxidative or flavoprotein enzymes The total free energy change for the conversion of β-cyclopiazonic acid into the proposed intermediate compound (dehydrogenated β-cyclopiazonic acid) should of course be negative Without studying the rate of the reverse reaction by employing the proposed intermediate (which is not available) as substrate it is impossible to determine this constant The fact that this compound may act rather as a substrate for the concerted cyclization reaction can make the determination of such a constant impossible

Amino acid composition of the isoenzymes of β-cyclopiazonate oxidocyclase

The amino acid compositions of the isoenzymes are given in Table V

The fact that the total amount of amino acid residues recovered from isoenzyme I and II was somewhat less than that from the other isoenzymes may be due to their 2–5% carbohydrate content Glucosamine and galactosamine could not be detected Due to lack of protein, hydrolysis could not be performed for periods longer than 24 h Single samples of isoenzyme III, however, were hydrolysed for 48 and 72 h, respec-

TABLE V

AMINO ACID COMPOSITION OF THE FIVE ISOENZYMES OF β-CYCLOPIAZONATE OXIDOCYCLASE (AMINO ACID RESIDUES/MOLECULE ENZYME)

Molecular weights taken as 51 000 for all the isoenzymes in the calculations of the number of amino acid residues/molecule of enzyme

Amino acid	*Amino acid residues/molecule of enzyme for the five isoenzymes (nearest integral)*				
	I	*II*	*III*	*IV*	*V*
Aspartic acid	48	43	45	44	40
Threonine	28	26	28	27	23
Serine	29	28	25	33	34
Glutamic acid	33	34	33	32	34
Proline	23	23	23	19	19
Glycine	33	34	32	35	37
Alanine	31	29	28	27	27
Valine	25	25	27	23	24
Methionine	3	2	2	4	3
Isoleucine	20	21	22	20	20
Leucine	28	30	33	30	29
Tyrosine	15	15	17	17	18
Phenylalanine	15	16	18	17	17
Lysine	19	20	21	24	26
Histidine	8	10	10	10	10
Arginine	14	17	18	17	17
Tryptophan	9	10	11	11	11
Half-cystine	5	–	3	–	–
Ammonia	35	32	44	76	74
Total (without NH_3)	386	383	396	390	389

tively The results obtained from these samples indicated that there was a decrease in the threonine and serine content but an increase of the valine and isoleucine content similar to that reported by MAYHEW AND MASSEY[12] for a flavodoxin purified by them Assuming that this finding was applicable to all the isoenzymes, correct values for these amino acids were obtained by extrapolation[12]

The most significant differences in the amino acid composition of the five isoenzymes were their basic and acidic amino acid content and their ammonia content A gradual increase in basic amino acids from isoenzyme I–V of 41 to 53 residues and in ammonia content from 32 to 76 residues and a gradual decrease in acidic amino acids from 81 to 74 residues per molecule of enzyme were found The decrease in acidic amino acids *minus* the ammonia residues per molecule (assuming that the ammonia is in the amide form in the protein) for enzyme I–V varied from 46 to 0 The above-mentioned results are in good agreement with and might offer an explanation for the previously observed differences in the magnitude of the positive charge of these isoenzymes[1]

Using the method described by BUCHANAN AND RABINOWITZ[10], the five isoenzymes were found to contain no acid labile inorganic sulphur Glucose oxidase[38,39] and L-amino acid oxidase[40] are examples of flavoprotein enzymes that also contain small quantities of carbohydrates The composition of the carbohydrates was not determined The function of such moieties in terms of the catalytic activity of isoenzymes I and II are still obscure, but their presence may be ascribed to previous association of these isoenzymes with the cell walls

Studies on the flavin prosthetic group of the β-cyclopiazonate oxidocyclases

The ultraviolet and visible spectra of the isoenzymes showed absorption peaks

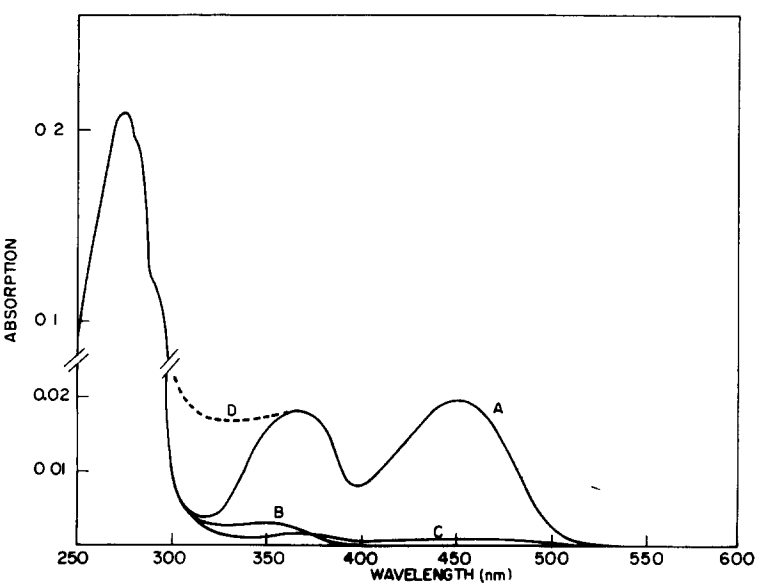


Fig 6 Absorption spectrum of isoenzyme III in 0 02 M sodium phosphate buffer (pH 6 7) (A) The protein concentration was about 0 2 mg/ml The absorption spectrum after the addition of a small excess of sodium dithionite (B) or β-cyclopiazonic acid (C) are also illustrated (D) shows the type of spectra given by isoenzymes I and II in the 340–350-nm region

at 276, 366 and 450 nm and a shoulder at 290 nm (Fig 6) The purified enzyme solutions had a yellow colour The addition of sodium dithionite or β-cyclopiazonic acid caused the disappearance of the 450 nm peak, due to the reduction of the flavin bound to the enzyme protein (Fig 6) α-Cyclopiazonic acid had no similar effect The results are similar to those reported for many flavoproteins[26,34,41,42] The absorption of isoenzymes I and II in the 340–350-nm region is higher in relation to the absorption peak at 360 nm than that found for the other isoenzymes In fact, their absorption spectra are in close agreement with that reported for milk xanthine oxidase by MACKLER *et al* [43]

The flavin residues released from the protein molecules showed absorption peaks at 450, 370 and 262 nm When activated at 450 nm the isoenzymes showed fluorescence peaks at 525–531 nm whereas the released flavin residues showed peaks at 517–519 nm (Fig 7) which are similar to the fluorescence peaks found for FAD and FMN under similar conditions (see EXPERIMENTAL PROCEDURES)

The fluorescences of the β-cyclopiazonate oxidocyclases are lower than that of the released flavin residue, probably as a result of a quenching of the fluorescence by the protein The fluorescence of the released flavin residue was also much lower than

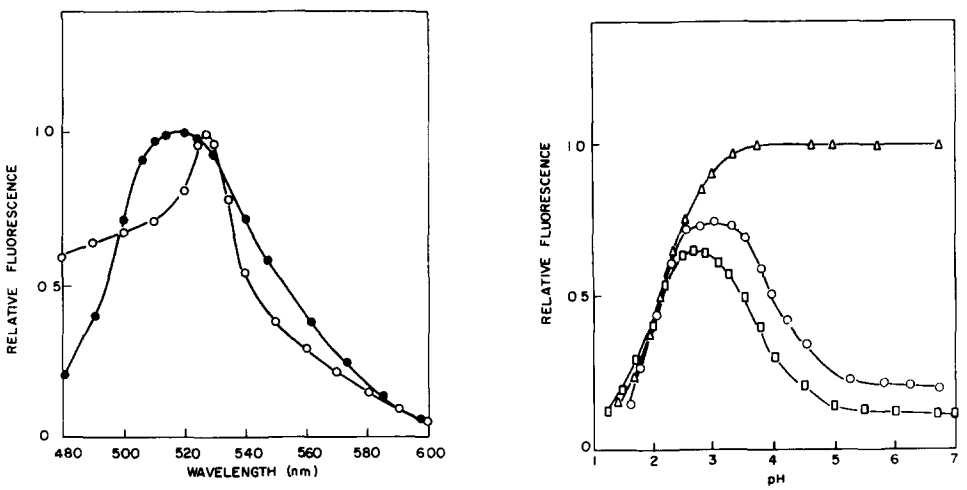


Fig 7 Fluorescence emission spectra of β-cyclopiazonate oxidocyclase (isoenzyme III) (○) and the flavin residue obtained from isoenzyme III by proteolytic digestion (●) in 0 05 M Tris–HCl buffer (pH 7 1) The enzyme concentration used was 250 μg/ml and the amount of flavin residue corresponded to 180 μg/ml isoenzyme III

Fig 8 The effect of pH on the fluorescence of FAD (□), FMN (△) and the flavin residue obtained by proteolytic digestion of isoenzyme III (○) corresponding to about 160 μg protein per ml

that of FMN at neutral pH However, the fluorescence increased enormously upon acidification An example of this characteristic pH dependence of the fluorescence of the flavin residues is illustrated in Fig 8, which includes for comparison the pH–fluorescence curves of FMN and FAD[44,45] It may be noted that on the acid side of pH 3 0 the pH–fluorescence curve of the flavin closely resembles those of FMN and FAD, but at pH values higher than 3 2 there is extensive internal quenching which is, however, less extensive than that of FAD in the pH range of 3 2–5 5 Purified flavin

residues obtained by paper chromatography followed by extraction or evaporation in a rotary evaporator gave similar results in fluorescence studies It thus appears as if amino acids and peptide residues of the protein produced by proteolytic digestion did not effect the fluorescence of this compound

Flavin residues obtained after two cycles of paper chromatography[15] employing either butanol–acetic acid–water or 5% Na_2HPO_4 (w/v) as solvent followed by extraction from the paper with water, were used for identification by paper chromatography employing both solvents When 5% Na_2HPO_4 was used as solvent the R_F value for the flavin corresponded with that of FAD, but in butanol–acetic acid–water it did not correspond with FAD or FMN Ninhydrin-reacting material was visualized by a very sensitive spraying method[15] Flavin residue spots were found to be ninhydrin-positive, which indicated that the flavin residues were probably still attached to an amino acid and/or small peptide This fact could explain the difference in the flavin residue R_F value in butanol–acetic acid–water and may also explain the internal fluorescence quenching of the flavin residues at pH 4 0 It can be concluded that in the native enzyme the flavin is bound by covalent linkage to the protein and that during digestion, proteolysis stops at points determined by the specificity of the pronase or by structural or steric hindrance by the flavin

Pentose determinations[9] on the flavin residues indicated that the flavins were dinucleotides

Flavin concentrations were calculated from the extinction coefficient of FAD at 450 nm, *viz* 11 3 M^{-1} cm^{-1} (see refs 46 and 47) It was calculated that the isoenzymes contain between 0 65 and 0 80 moles of flavin released per mole of enzyme (mol wt taken as 50 000) Longer periods of digestion with pronase did not yield higher values for the flavin content and pronase digestion of the protein residues obtained by thermal coagulation of the original digests showed no detectable quantities of flavin Because it is quite possible that less than 100% of the bound flavin might have been released by pronase digestion, it may be assumed that the enzymes contain 1 mole of flavin (most probably FAD) per mole of enzyme

CONCLUSIONS

Results obtained in studies on the reactions catalysed by the β-cyclopiazonate oxidocyclases indicate that the conversion of β-cyclopiazonic acid into α-cyclopiazonic acid occurs in two concerted steps The first reaction is a dehydrogenation reaction with the formation of a double bond Several examples of flavoprotein-catalysed double bond formations are known

The cyclization reaction or second reaction appears to be in concert with the dehydrogenation reaction and may also be catalysed by the enzyme, as indicated by the TPCK inhibition

According to results obtained from preliminary studies of the effect of pH on the activity of the isoenzymes and the inhibition of the dehydrogenation reaction by DNFB, it seems that an imidazole group of histidine and an α-amino group may be involved in the breakdown of the enzyme-substrate complex The mechanism of the dehydrogenation reaction can be regarded as principally similar to the mechanism described for dehydrogenations like the conversion of succinic acid to fumaric acid[48,49] and 3-oxo steroids into $\Delta^{1,4}$-3-oxo steroid[50,51] This two-step mechanism involves the

initial removal of a proton from the carbon atom adjacent to an electron-withdrawing group (in this case the electron-withdrawing group is at the end of a conjugated system), followed by a hydride transfer to a suitable oxidizing agent

Seeing that the cyclization reaction appears to take place in concert with the dehydrogenation reaction and that the proposed intermediate may be unstable, it can be concluded that the substrate stays bound to the same site on the enzyme molecule during the dehydrogenation as well as the cyclization reaction It can be suggested that the cyclization reaction is catalysed by a neighbouring positively charged group on the enzyme molecule Such a positive group could induce a positive charge at the b position of the proposed intermediate compound[1] (see Fig 9) by means of relating either one of the two conjugated systems to the specific position of the double bond in the intermediate The cyclization reaction can then be initiated by a nucleophilic attack by the π-electrons of the double bond in the mevalonate-derived C_5-unit (in position 4 of the indole) on this positively charged, b position carbon atom followed

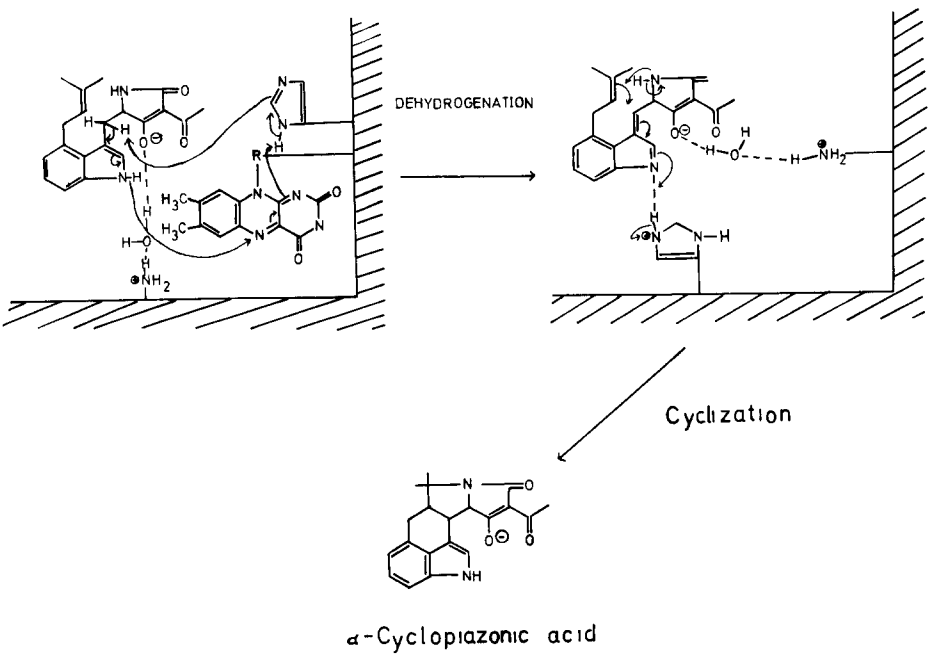


Fig 9 Proposed mechanism for the dehydrogenation and cyclization reactions in the conversion of β-into α-cyclopiazonic acid by the β-cyclopiazonate oxidocyclases

by a concerted movement of the lone pair of electrons on the nitrogen, as indicated in Fig 9 Reasoning from the inhibition of the cyclization reaction by TPCK, it seems that the positively charged groups may be an imidazole group but may as well be a lysine ε-amino or α-amino group We will attempt to determine the exact position of the double bond in the proposed intermediate in the near future and to obtain more definitive evidence that the cyclization reaction is also catalysed by the enzyme

This cyclization reaction differs from that catalysed by 2,3-oxidosqualene lanosterol cyclase in that it appears to take place in concert with an oxidative step, where-

as squalene cyclization is catalyzed by an enzyme different from the oxidative one which catalyses the formation of oxidosqualene[18]

Although the postulated mechanisms may be oversimplified, they clearly indicate that these reactions can be explained in terms of molecular mechanisms

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