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EXTRACTION AND PROPERTIES OF ALDEHYDE DEHYDROGENASE FROM *PSEUDOMONAS AERUGINOSA*

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SUMMARY

1. An aldehyde dehydrogenase (aldehyde:NAD(P) oxidoreductase) from a strain of *Pseudomonas aeruginosa* (Sol 20) grown with paraffin hydrocarbons as sole carbon source was obtained and partially purified from cell-free extracts.

2. The aldehyde dehydrogenase was specific for aliphatic aldehydes and required the presence of Fe^{2+} or Ca^{2+}, and a flavin such as FAD or riboflavin.

3. The action of compounds having –SH groups established that CoA and mercaptoethanol were activators of the enzyme.

4. The aldehyde dehydrogenase was only found in preparations from bacteria grown on higher alcohols, aliphatic acids or hydrocarbons. This confirms that the aldehyde is an intermediate in the metabolic degradation of hydrocarbons by bacteria.

INTRODUCTION

During earlier work it was shown[1] that a *Pseudomonas aeruginosa* strain (Sol 20) degraded paraffin hydrocarbons by a process in which the intermediate stages were the primary alcohol, aldehyde, and fatty acid corresponding to the initial substrate. Furthermore[2], enzymic extracts of bacteria grown on *n*-heptane contained an alcohol dehydrogenase (alcohol:NAD oxidoreductase) particularly active with higher alcohols and an aldehyde dehydrogenase (aldehyde:NAD(P) oxidoreductase) with heptaldehyde as substrate. The partial purification of the former established that these two enzymes were different. The present work was undertaken to isolate the aldehyde dehydrogenase and study its properties.

METHODS

The bacteria were grown in a mineral medium containing *n*-heptane as sole carbon source, incubated at 32° with vigorous shaking and harvested as previously described[3]. The enzymic extracts were prepared from suspensions of bacteria in 0.05 M Tris

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buffer (pH 8.5) by treatment for 5 min in Raytheon 10 kcycles sonic disintegrator. The cellular debris was separated by centrifugation at 18 000 rev./min for 20 min in an International refrigerated centrifuge, model HR 1. The dehydrogenase activity of the cell-free extracts was established by reduction of nicotinamide nucleotides and observed at 340 mμ in a Jobin and Yvon spectrophotometer with systems of the following composition, incubated at 24°: 7 mM NAD, 0.3 ml; 5 mM heptaldehyde suspension, 0.3 ml; 0.3 ml enzymic extract (equivalent to about 1–1.5 mg protein nitrogen); 0.05 M glycine–NaOH buffer (pH 9.4) to a total volume of 3.0 ml.

The suspension of heptaldehyde was obtained by treatment in the sonic disintegrator of an aqueous mixture containing 500 μg pure heptaldehyde (Eastman-Kodak) per ml. It was essential to add this substrate last to the experimental mixture, followed by vigorous agitation. The enzymic activity of the aldehyde dehydrogenase is expressed in mμmoles of NAD reduced per min per mg protein nitrogen.

Partial purification of the enzyme

The crude extracts obtained in the conditions described above were partially purified by using the modified technique of JAKOBY[4]. The crude extracts had a specific activity of 530 units/mg protein nitrogen or 90 units/mg protein.

Crude extract (100 ml) was first subjected to a fractionation with $(NH_4)_2SO_4$: a first fraction, precipitated between 0 and 30% satn. was discarded. The supernatant fluid was again treated with $(NH_4)_2SO_4$ to 55% satn. and the precipitate recovered by centrifugation (29 000 $\times$ g at 4° for 10 min) and dissolved in 0.05 M Tris buffer (pH 8.5) containing 0.02 M mercaptoethanol.

The purification was continued by precipitation with acetone at 0°. Acetone (33 ml) was added over a period of 10 min to the 40 ml redissolved precipitate from the $(NH_4)_2SO_4$. The precipitate was rejected and to the supernatant fluid were added with stirring a further 7 ml of acetone during 5 min.

The precipitate recovered by centrifugation at 0° was dissolved in 10 ml 0.05 M Tris buffer (pH 8.5) plus 0.02 M mercaptoethanol to which was added an equal volume of satd. $(NH_4)_2SO_4$ containing 5% (v/v) of conc. ammonia solution (s.g. 0.880). After leaving overnight at 0° the mixture was centrifuged, the precipitate rejected and the supernatant fluid brought to 100% satn. with $(NH_4)_2SO_4$ and recentrifuged.

The precipitate, taken up in 5 ml 0.05 M Tris buffer (pH 8.5) plus 0.02 M mercaptoethanol, constituted the last fraction, of which the specific activity was about 14 times that of the initial crude extracts, *i.e.* about 1260 units/mg protein.

During this purification the enzymic losses were significant and the final yield small. However, the final preparation was totally devoid of alcohol dehydrogenase.

RESULTS

Properties of the crude extracts

As already described[2] for the alcohol dehydrogenase of the same strain of *P. aeruginosa*, only organisms grown on higher alkanes or aldehydes could take up O_2 in the Warburg apparatus in the presence of heptaldehyde. In parallel with this, only cell-free extracts from bacteria grown in these conditions possess an aldehyde

dehydrogenase activity. However the mode of preparation of the enzymic extracts influenced their activity. Contrary to the results obtained with alcohol dehydrogenase, the use of 0.05 M phosphate buffer (pH 7.1) gave an activity lower by half than that in Tris buffer (pH 8.5). These enzymic preparations were rather unstable, and the best conditions for stability (0.05 M Tris buffer (pH 8.5) plus 0.02 M mercaptoethanol) still resulted in a 50% loss of activity in 5 days.

In the presence of a defined quantity of heptaldehyde (0.5–1 μmole/ml) the reduction of NAD by the extracts was linear as a function of time. This activity was not specific for NAD, which could be replaced by NADP. The specific activity of crude extracts as determined in the indicated conditions varied between 400 and 700 units when NAD was used as hydrogen acceptor; with NADP this activity was about halved.

It is necessary to take into consideration the fact that in the experimental con-

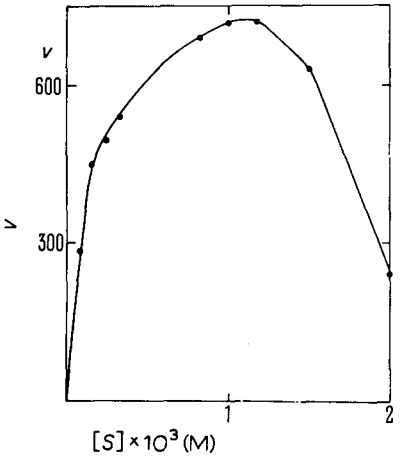

Fig. 1. Oxidation of heptaldehyde as a function of substrate concentration. V, mμmoles NAD reduced per min per mg protein nitrogen. S, concentration of heptaldehyde in the system. Maximum at 1.16 mM.

ditions used, the crude extracts contained an alcohol dehydrogenase in addition to the aldehyde dehydrogenase, and were thus able to bring about the reaction: heptaldehyde + $NADH_2$ → heptanol + NAD which might interfere with the dehydrogenation of the aldehyde. However if one refers to the relative activities of the two dehydrogenases one finds that the aldehyde dehydrogenase was 8–9 times as active as the alcohol dehydrogenase for which, furthermore, the reverse reaction proceeds at one-tenth of the velocity of the forward reaction under the same experimental conditions[2]. It appears that this interference cannot therefore be significant.

The speed of reduction of NAD and NADP was proportional to the amount of extract; for a given quantity of enzyme preparation the rate (Fig. 1) increased with heptaldehyde concentration, reaching a maximum value at a substrate concentration of 1.16 mM. At higher concentrations the substrate appeared to inhibit strongly, so that at 2 mM the rate was no more than 20% of the maximum.

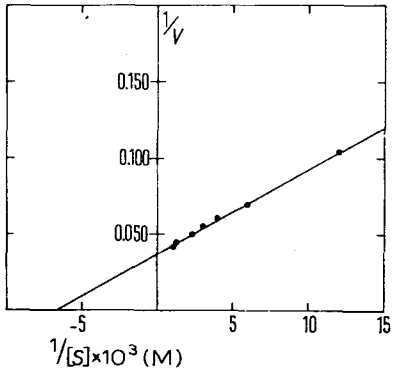

Fig. 2. Lineweaver–Burk plot relating heptaldehyde concentration to initial rate of oxidation. $\frac{-1}{K_m} = 6.6 \cdot 10^3$; whence $K_m = 0.15$ mM.

The numerical value of the Michaelis constant (Fig. 2) for heptaldehyde was 0.15 mM, very close to the value calculated by JAKOBY for the aldehyde dehydrogenase of *P. fluorescens* with respect to glyceraldehyde[4]. For electron acceptors the K_m values were 0.3 mM for NAD and 4 mM for NADP.

The dehydrogenation of heptaldehyde in crude extracts did not require the presence of phosphate or arsenate. However, the rate was increased by 25–30% when 1 mM ATP was added, by 17% in presence of 1 mM Fe^{2+}, and by 20% when Fe^{2+} was replaced with Ca^{2+}.

The product formed in the course of the dehydrogenation of heptaldehyde was identified as the corresponding free fatty acid, heptanoic acid. This compound was demonstrated by the use of infrared spectroscopy after a technique already shown[5] in the study of the formation of hept-1-ene from *n*-heptane.

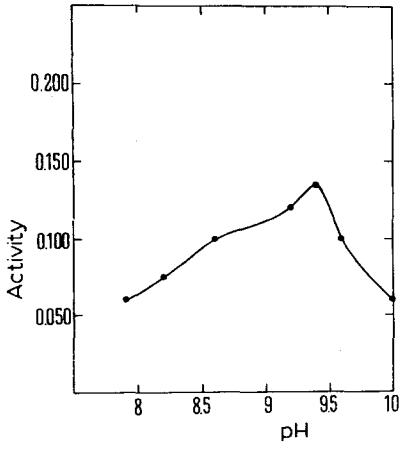

Fig. 3. The solid line represents the rate of heptaldehyde oxidation as a function of pH. pH 7–8.5: Tris buffer; pH 8.5–10: glycine–NaOH buffer. Maximum at pH 9.4.

An experimental system containing the necessary constituents for the enzymic dehydrogenation of heptaldehyde in 5 times the above described quantities was extracted with CCl_4 (3 ml) and H_2SO_4 at the end of the reaction, that is, when all the NAD seemed to have been reduced to $NADH_2$. The qualitative and quantitative analysis of this CCl_4 extract showed the appearance of free heptanoic acid in quantity equal to that of the NAD enzymically reduced. By comparison, the analysis of a control system identical but for the absence of NAD, showed that the heptaldehyde could be quantitatively recovered and that no trace of fatty acid, free or esterified, could be detected in it. Furthermore, the analysis of a sample of CCl_4 from an experimental system identical but for the addition of 0.05 M CoA and 1 mM ATP showed the recovery of the same quantities of free acid, again without trace of esters.

The aldehyde dehydrogenase activity was optimal (Fig. 3) at pH 9.4 in 0.05 M glycine–NaOH buffer. For the same pH value (pH 8), 0.05 M pyrophosphate buffer stimulated by 40–45% and 0.05 M phosphate buffer inhibited by 10–20%. In presence of 0.05 M borate buffer, the enzyme was completely inactive.

The enzyme activity was completely destroyed by heating to 100° for 5 min, lost 87% of activity on heating to 55° for 3 min or 60% on heating to 45° for 3 min Optimal enzymic activity was at 26–27°.

Action of compounds with sulfhydryl groups on the aldehyde dehydrogenase activity in crude extracts

Sulfhydryl compounds had a particular action on the aldehyde dehydrogenase activity of crude extracts of *P. aeruginosa* grown on hydrocarbons. The partial purification of these extracts required presence of 0.02 M mercaptoethanol. This phenomenon has already been reported by JAKOBY[4,6]. However, in the present case the results differ according to the nature of the compound used.

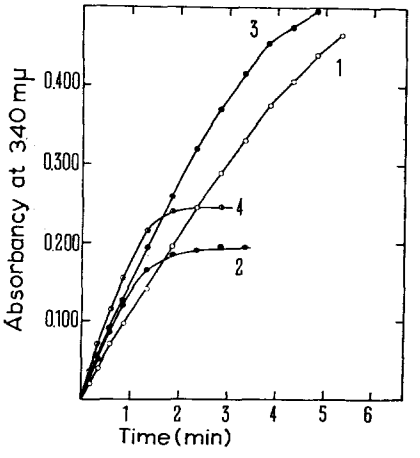

Fig. 4. Study of the speed of reduction of NAD in 3-ml systems containing crude enzymic extract (0.3 ml), NAD (7 mM, 0.3 ml), heptaldehyde (5 mM, 0.3 ml), glycine–NaOH buffer (pH 9.4) and additions as follows: 1, no addition; 2, cysteine·HCl (0.1 mM, 0.3 ml); 3, sodium arsenite (1 mM, 0.3 ml); 4, sodium arsenite (1 mM, 0.3 ml) and cysteine·HCl (0.1 mM, 0.3 ml).

Mercaptoethanol at 1 μM stimulated the enzymic activity by 14%, and at 3 μM by 33%. CoA likewise stimulated, by 50–60% at 0.1 mM, but reduced glutathione inhibited by about 43–50% at 0.3 mM. 10^{-5} M cysteine·HCl added to the reaction system at the same time as the substrate gave first an activation of 25%, after 1 min the enzymic reaction was totally inhibited (Fig. 4).

When, for a particular time, the amount of NAD reduced in the normal system is compared with that in similar systems with increasing amounts of cysteine·HCl it is found that for a concentration of the latter equal to 10^{-5} M, only 65% of the amount of NAD was reduced to $NADH_2$, for $2 \cdot 10^{-5}$ M only 54% and for $3 \cdot 10^{-5}$ M only 41%. Pre-incubation with cysteine for 5 min nullified the activity of the enzyme.

The action of sodium arsenite on this enzymic activity may be compared with that of sulfhydryl compounds. Contrary to the findings of JAKOBY[6] with *P. fluorescens* and of other authors with the pyruvate oxidase system[7,8], sodium arsenite at 0.1 mM activated the aldehyde dehydrogenase by 37% (Fig. 4).

When 0.1 mM sodium arsenite was added at the same time as 10^{-5} M cysteine a stimulation was found of 75% compared to the normal system, or of 50% compared to the system with cysteine only. The total quantity of NAD reduced to $NADH_2$ was also greater than in the system with cysteine alone, without, however, reaching that of the system without cysteine.

Neither lipoic acid nor cystine had any action on the aldehyde dehydrogenase.

Inhibitors

The enzymic activity was completely inhibited (Table I) by *p*-chloromercuribenzoate, borate, Cu^{2+} and Zn^{2+}, even at concentrations of the order 0.1 mM.

Unlike the alcohol dehydrogenase, the aldehyde dehydrogenase was sensitive to sodium iodoacetate, and inhibition by KCN was much more marked, even at relatively low concentrations.

The enzymic reduction of NAD in presence of heptaldehyde was sensitive to complexing agents, such as 2,2'-dipyridyl and EDTA. Further, dialysis of the crude

TABLE I

INFLUENCE OF VARIOUS INHIBITORS ON THE ALDEHYDE DEHYDROGENASE ACTIVITY OF THE EXTRACTS

Inhibitor	*Concentration (M)*	*Inhibition (%)*
p-Chloromercuribenzoate	10^{-4}	100
p-Chloromercuribenzoate	10^{-5}	25
Borate	10^{-3}	90
Hydroxylamine	10^{-3}	14
KCN	$3 \cdot 10^{-4}$	60
2-2'-Dipyridyl	10^{-3}	30
EDTA	$3 \cdot 10^{-4}$	14
EDTA	$6 \cdot 10^{-4}$	43
Iodoacetate	$3 \cdot 10^{-4}$	20
Reduced glutathione	$3 \cdot 10^{-4}$	47
Zn^{2+}	$3 \cdot 10^{-4}$	100
Cu^{2+}	$3 \cdot 10^{-4}$	100

extract against distilled water with 5 mM Tris buffer (pH 8.1) or 5 mM glycine–NaOH buffer (pH 9) resulted in a 70% loss of activity; dialysis against 5 mM Tris buffer (pH 8.1) containing 0.1 mM EDTA resulted in the loss of 90% of the initial activity of crude extract. The activity of these dialysis residues was restored partially by Fe^{2+} or Ca^{2+} (50% reactivation) or by FAD or riboflavin (about 40% reactivation). FMN and salts of magnesium or manganese had no action on the same dialysis residues.

Properties of the partially purified extracts

The specific activity of the partially purified enzyme was maximum for heptaldehyde and octaldehyde, smaller for the other aliphatic aldehydes and no more than 10% of the optimum with benzaldehyde, the only non-aliphatic aldehyde for which

TABLE II

RELATIVE ACTIVITY OF PARTIALLY PURIFIED ALDEHYDE DEHYDROGENASE WITH VARIOUS ALDEHYDES

Aldehyde (0.1 mM)	*Relative activity*
Heptaldehyde	100
Hexaldehyde	90
Octaldehyde	85
Acetaldehyde	40
Butyraldehyde	30
Decylaldehyde	20
Dodecylaldehyde	15
Benzaldehyde	10
DL-Glyceraldehyde	0
Glyoxal	0
Succinic semi-aldehyde	0

an activity could be demonstrated. The value of this activity was zero for DL-glyceraldehyde, glycolaldehyde, glyoxal and succinic semi-aldehyde.

The specificity of this enzyme is thus relatively narrow. Furthermore, partially purified extracts from organisms grown on dodecane or tetradecane always had the same ratio of specific activity for heptaldehyde and dodecaldehyde.

The partially purified extracts were quite incapable of performing the reverse reaction: heptanoic acid → heptaldehyde with either $NADH_2$ or $NADPH_2$, either in the conditions of pH value and concentrations of the forward reaction or in different conditions tested.

DISCUSSION

The partial purification and the study of the properties of the aldehyde dehydrogenase isolated from a strain of *P. aeruginosa* (Sol 20) grown on hydrocarbons show that this enzyme differs from the aldehyde dehydrogenase of *P. fluorescens* described by JAKOBY[4] and that of bakers' yeast studied by BLACK[9]. The aldehyde dehydrogenase

of *P. aeruginosa* has a specificity which is fairly strict for aliphatic aldehydes, but less so for the chain length, though maximum activity is obtained with heptaldehyde.

This enzyme is only poorly stable, and is very sensitive to complexing agents. It required the presence of divalent metals such as Fe^{2+} or Ca^{2+} as well as certain flavins (*e.g.* FAD, riboflavin).

The aldehyde dehydrogenase activity studied in the above conditions, though comparatively greater than the activity of alcohol dehydrogenase in the same cell-free extracts was still further augmented by the addition of CoA or ATP. This phenomenon suggests that during the dehydrogenation of the aldehyde there is formation of an acyl phosphate or acyl-CoA, probably as the intermediate of one or several other enzymes. At the same time these esters must be rapidly transformed to the free fatty acid by some agency, which would explain why the final product of heptaldehyde dehydrogenation is heptanoic acid. This is especially interesting as, apart from mercaptoethanol, all the other sulfhydryl compounds studied proved to be inhibitors of this dehydrogenase activity, and the enzyme can be effectively protected against these inhibitors by the addition of low concentrations of arsenite, ordinarily considered to be an inhibitor of aldehyde dehydrogenase activity. The lability of the present enzyme, its activation and its protection by mercaptoethanol and finally its activation by sodium arsenite suggest that in these preparations the aldehyde dehydrogenase may be bound to another enzyme which requires CoA and ATP and for which certain free sulphur compounds present in the enzymic extracts, such as cysteine and reduced glutathione are competitive inhibitors. The state of the results and experiments did not permit a categorical statement about this. Besides this hypothesis there is the experimental fact that the presence of significant quantities of free acids has been established in culture media of these bacteria on hydrocarbons.

In other ways it has been shown that other microorganisms able to grow at the expense of hydrocarbons give cell-free extracts which have an aldehyde dehydrogenase activity, *e.g.* a strain of *Candida lipolytica* and another strain of *P. aeruginosa* (Strain A). It seems that these aldehyde dehydrogenases may be identical in nature to that described above.

The preparations from *P. aeruginosa* (Strain A) grown on dodecane had aldehyde dehydrogenase activities varying from 2200 to 2900 units. This activity was specific for NAD, and could be completely recovered from the particles which sedimented in the Spinco ultracentrifuge between 20 000 and 40 000 rev./min. Moreover, the enzymic activity reached a plateau value between pH 8.5 and pH 9.4.

It is thus probable that the aldehyde dehydrogenase of *P. aeruginosa* (Strain A), differs from that isolated from *P. aeruginosa* (Sol 20). Nevertheless the above results show that aldehyde dehydrogenase is an inducible enzyme which only appears in organisms when they were grown on higher alcohols, aldehydes, or paraffins. This supports the conclusion already proposed[1] that the aldehyde be included in the metabolic scheme of the bacterial degradation of paraffin hydrocarbons.

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