On a non-pyridine nucleotide-dependent 2-oxo-acid reductase of broad substrate specificity from two *Proteus* species

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Proteus mirabilis and *Proteus vulgaris* contain in the crude extract a non-pyridine nucleotide dependent reductase for 2-oxo-acids as well as 2-oxo-dicarboxylic acids of specific activities of 1–10 units/mg protein. The enzyme catalyzes the reduction of very different 2-oxo-acids to the corresponding (2R)-hydroxy-acid. Reduced methyl- or benzylviologen act as artifical electron donors. The membrane-bound enzyme has been enriched 167-fold to a purity 90% as judged by electrophoresis. It seems that the enzyme does not contain flavin.

Proteus mirabilis Proteus vulgaris *2-Oxo-carboxylic acid reductase* *(2R)-Hydroxy-acid preparation* *Viologen*

1. INTRODUCTION

Most carbonyl group reducing enzymes are pyridine nucleotide-dependent, which means they belong to the subgroup EC 1.1.1 of the enzyme nomenclature. Enzymes subsumed under EC 1.1.2 and 1.1.99 usually act only as dehydrogenases. Well known 2-oxo-acid reductases are the lactate dehydrogenases which reveal a rather confined substrate specificity [1–3]. We observed an astonishingly high activity of a 2-oxo-acid reductase in *Proteus mirabilis* and *Proteus vulgaris* for which NADH and NADPH do not act as electron donors in the presence of 2-oxo-acids. However, the enzyme catalyzes the strict stereospecific reduction of a surprisingly broad spectrum of 2-oxo-carboxylates and 2-oxo-dicarboxylates with reduced methyl- or benzylviologen as electron donors. Besides this interesting feature, the enzyme is rather versatile from a preparative point of view; i.e., the synthesis of (2R)-hydroxy-acids.

Abbreviations: methylviologen or MV^{2+}, 1,1′-dimethyl-4,4′-dipyridinium dication; $MV^{+\cdot}$, reduced methylviologen; BV^{2+}, benzylviologen; $BV^{+\cdot}$, reduced benzylviologen

To the best of our knowledge a similar reductase seems not to be known. Therefore we report on its substrate specificity and enrichment.

2. MATERIALS AND METHODS

The 2-oxo-acids were gifts from Degussa AG (D 6000 Frankfurt) or bought from commercial sources. Solutions of 5 mM methyl- and benzylviologen hydrochlorides (Sigma, 8000 Munich) in 100 mM phosphate buffer (pH 7.0) were electrochemically reduced at −790 and −620 mV, respectively, against a standard calomel electrode and stored in Schlenk-Tubes under purified nitrogen. *P. mirabilis* and *P. vulgaris* were obtained from Deutsche Sammlung von Mikroorganismen as DSM 30 115 and DSM 30 118, respectively.

The enzyme was assayed under strict anaerobic conditions in cuvettes closed with rubber stoppers. The cuvettes were repeatedly evacuated and refilled with nitrogen. A total volume of 2.1 ml contained 100 mM phosphate buffer (pH 7.0), 0.4 mM reduced viologen, 2 mM 2-oxo-carboxylate and about 10 munits of the enzyme. The reaction was followed by the decrease of the absorption at 578 nm (ϵ_{578} = 12 $cm^2 \cdot mmol^{-1}$ for reduced benzyl-

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viologen and 9.0 for reduced methylviologen, respectively) in an Eppendorf photometer. One unit is defined as the reoxidation of 2 μmol reduced viologen per min in the presence of 2 mM phenylpyruvate.

Growth medium: 1 l of deionized water contained 5 g yeast extract (Difco), 5 g glucose, 5 g K_2HPO_4 and 20 g peptone (Merck, D-Darmstadt) or Tryptone (Oxoid).

P. vulgaris was grown between 0.5 l and 300 l at 37°C under anaerobic conditions. After 18 h usually an absorbance of 1.7 measured at 578 nm was reached. *P. mirabilis* showed increased activities when grown under bubbling with 3% oxygen and 97% nitrogen. After 8 h an absorbance of 2.4 was observed. *P. mirabilis* cultures were inoculated with a preculture of 1% volume and *P. vulgaris* with 0.5%, respectively.

Enrichment procedure (see table 1): if not stated otherwise all procedures were carried out at 0–4°C. Wet packed cells (50 g) suspended in 150 ml 20 mM phosphate buffer (pH 7.0) containing 0.5 mM phenylpyruvate were sonicated for 45 min. After centrifugation of the lysate for 15 min at 13 000 × *g* the supernatant was again centrifuged at 100 000 × *g* for 120 min. The sediment was washed with 105 ml 20 mM phosphate buffer and then extracted with the same buffer (pH 7.0) containing 1% Triton X-100 by stirring for 45 min in an ice bath, again centrifuged for 75 min at 100 000 × *g* and concentrated to 30 ml by ultrafiltration using a YM-30 Amicon filter. This solution was chromatographed on a Sepharose-6B column (85 × 5 cm) by applying a 20 mM phosphate buffer (pH 7.0) containing 0.5 mM phenylpyruvate, 0.5 mM dithioerythritol and 0.05% Triton X-100. The latter 3 components were included in all buffers, if not stated otherwise. The activity-holding fractions were collected, concentrated by ultrafiltration and finally brought to a volume of 35 ml in 10 mM phosphate buffer. This solution was separated on a hydroxylapatite (Bio-Gel) column (18 × 2.5 cm) equilibrated with 10 mM phosphate buffer. The column was eluted with the equilibration buffer. The enzyme containing fractions were combined and the buffer was changed to 10 mM Tris–HCl (pH 7.5) by dia-ultrafiltration. This solution was put on a DEAE–Sephadex A-25 column (18 × 1.5 cm) and eluted with a linear gradient of 0–700 mM KCl in the aforementioned buffer.

Electrophoresis was carried out with system 1 as in [4]. The analysis for flavin was conducted as in [5] and [6].

3. RESULTS AND DISCUSSION

The reductase catalyzes the reaction:

$$RCOCOO^- + 2MV^{+\cdot} \text{ (or } 2\ BV^{+\cdot}) + 2H^+ \rightarrow RCHOHCOO^- + 2MV^{2+} \text{ (or } 2BV^{2+})$$

So far it has not been possible to demonstrate the reverse reaction, even in the presence of ferricyanide together with oxidized viologens. The former should reoxidize trace amounts of reduced viologens in order to shift the thermodynamically unfavorable back reaction.

As shown elsewhere the (2R)-hydroxycarboxylates obtained from the corresponding 2-oxo-acids by the hydrogenation with cells of *P. mirabilis* are enantiomerically pure in the limits of rather sensitive methods [7].

The enzyme content in the crude extract varies with the growth medium and conditions from 0.2 to 2 units/mg protein in *P. mirabilis* and from 0.6 to 10 units/mg protein in *P. vulgaris*, respectively.

Table 1 shows an enrichment procedure for the 2-oxo acid reductase from *P. mirabilis*. The 3 indicated column separations lead to a preparation with a purity of 90% as judged by electrophoresis. After the described ultracentrifugations, the enzyme is free from hydrogenase and 2-oxo-acid decarboxylase activities. Therefore such a preparation can be used for preparative purposes, for instance, in the electro-enzymatic fashion [7–9]. The spectrum of the purified enzyme as shown in fig.1 indicates a characteristic maximum at 418 nm. This maximum increases proportionally with the specific activity of the enzyme. The flavin content is lower than 1 mol/200 000 × g enzyme. Therefore, we assume that the reductase is not a flavoenzyme. So far we do not know which group causes the absorption at 418 nm and 318 nm, respectively. Especially the absorption at 418 nm is somewhat reminiscent of the heme-containing enzymes such as *Pseudomonas* cytochrome oxidase [10], nitrite reductase from *Achromobacter fisheri* [11], or adenyl sulfate reductase from *Thiocapsa roseopersicina* [12]. However, the aforementioned 3 enzymes are all inhibited by cyanide as well as by

Table 1

Purification of 2-oxo-acid reductase from *Proteus mirabilis*

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Enrichment (-fold)	Yield (%)
Crude extract	4004	7280	1.8	–	100
Ultracentrifugation, extraction, ultracentrifugation and ultrafiltration	407	5500	13.5	7.5	76
Gelfiltration and ultrafiltration	168	4550	27.1	15	63
Hydroxylapatite and ultrafiltration	18.5	1300	70.3	39	18
DEAE-Sephadex A-25 and ultrafiltration	2.5	738	300	167	10

carbon monoxide. The 2-oxo-acid reductase shows no inhibition with carbon monoxide and only 30% inhibition with 10 mM cyanide. Especially surprising is the broad substrate specificity of the enzyme from *P. mirabilis* as well as that from *P. vulgaris* (table 2). Not only 2-oxo-monocarboxylates are substrates but also 2-oxo-dicarboxylates. Branching in the 3-position of a substrate leads to diminished reduction rates. This can be seen from the comparison of 2-oxo-4-methylpentanoate with 2-oxo-3-methylpentanoate and 2-oxo-3-dimethyl-4-hydroxy-butanoate. Phenylglyoxylate also behaves according to this rule. There seems to be

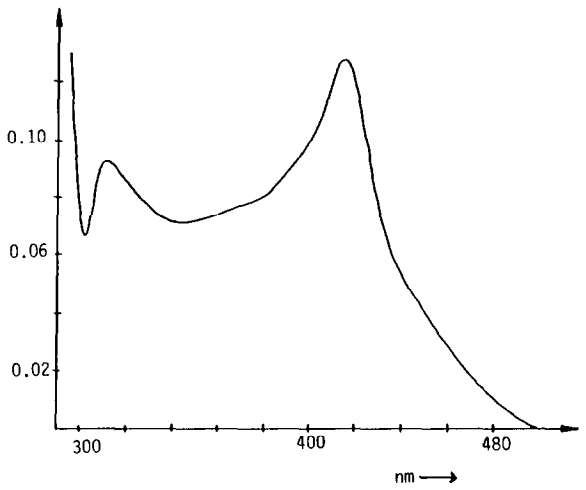

Fig. 1. Absorption spectrum of the 2-oxo-acid reductase from *P. mirabilis* in a 1-cm cuvette after 167-fold enrichment and about 90% purity as judged by electrophoresis. Protein concentration 0.3 mg/ml in 20 mM phosphate buffer.

Table 2

Relative rates of the reduction of 2-oxo-acids to hydroxy-acids with partially purified 2-oxo-acid reductase from *P. mirabilis* as well as from *P. vulgaris* and $MV^{+\cdot}$ or $BV^{+\cdot}$

Substrate	Relative activity (%)	
	P. mirabilis	*P. vulgaris*
Phenylpyruvate[a]	100	100
Pyruvate[a]	92	85
Indolylpyruvate	35	67
5-Benzyloxyindolyl-pyruvate	30	–
3-Fluoropyruvate	22	20
4-Oxo-4-methyl-pentanoate	81	–
(S)-2-Oxo-3-methyl-pentanoate	28	25
(R,S)-2-Oxo-3-methyl-pentanoate	–	46
2-Oxo-3,3-dimethyl-4-hydroxybutanoate[a]	7	4
Phenylglyoxylate[a]	16	5
2-Oxo-nonanoate	83	–
Oxalacetate	73	50
2-Oxoglutarate	78	62
2-Oxoadipate	70	–
2-Oxo-(4-hydroxy-methyl-phosphinyl)-butanoate	24	–
3-Oxoglutarate	0	–
Hydroxyacetone	0	–

[a] The products of these substrates have been checked for optical purity. Within the limits of experimental error they turned out to be pure (2R)-hydroxy acids

a pronounced influence of the stereochemical configuration of C-3 as can be seen from the reduction rates of (R,S)-2-oxo-3-methylpentanoate and (R)-2-oxo-3-methylpentanoate. Reduced methylviologen reacts spontaneously with phenylglyoxylate. Therefore this substrate can only be studied with reduced benzylviologen. Use of the latter reagent leads via electro-enzymatic reduction [7-9] to (R)-mandelic acid with the correct optical rotation.

The rates observed with reduced methylviologen as well as reduced benzylviologen and phenylpyruvate are similar as long as the concentrations of the viologens are about 0.1 mM.

The enzyme activity stays constant for at least 30 h when used for the electro-enzymatic reduction of (2R)-hydroxy acids in a preparative scale at room temperature [8].

In general, preparative scale reductions cannot be performed using reduced pyridine nucleotide in stoichiometric amounts due to their high cost. Therefore, an enzyme such as the 2-oxo-acid reductase has advantages over pyridine nucleotide-dependent reductases mainly for two reasons: (i) reduced methylviologen can be regenerated electrochemically in contrast to NAD(P)H which need for their regeneration a second enzyme and a second substrate [13,14]; (ii) methylviologen is chemically and enzymatically more stable than pyridine nucleotides.

So far the physiological role of this apparently membrane-bound reductase is not known.

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