Volume 266, number 1,2, 183-186 FEBS 08535 June 1990

Purification and some characteristics of chicken liver L-2-hydroxyacid oxidase A

Liliane Dupuis, Josiana De Caro, Patrick Brachet and Antoine Puigserver

Centre de Biochimie et de Biologie Moléculaire du Centre National de la Recherche Scientifique, BP 71, 13402 Marseille Cedex 9, France

Received 9 April 1990; revised version received 26 April 1990

The isozyme A of L-2-hydroxyacid oxidase is a peroxisomal flavoenzyme that catalyzes the oxidation of short-chain aliphatic L-2-hydroxyacids in many tissues of higher organisms. A new purification procedure allowed us to obtain a 1400-fold purified enzyme from chicken liver. The N-terminal amino acid of the polypeptide chain was found to be blocked as that of spinach glycolate oxidase, contrastingly with that of rat kidney isozyme B. Its amino acid composition was comparable to that of other known L-2-hydroxyacid oxidases. Despite different substrate specificity, some immunological identity was observed between chicken liver L-2-hydroxyacid isozyme A and rat kidney isozyme B.

L-2-Hydroxyacid oxidase; Isozyme A; Glycolate oxidase; Peroxisome; Chicken liver

1. INTRODUCTION

The FMN-containing enzyme L-2-hydroxyacid oxidase (L-2-HAOX) is responsible for the oxidation of a number of L-2-hydroxyacids to the corresponding ketoacids with concomitant consumption of molecular oxygen and release of hydrogen peroxide. The enzymic activity has been detected in several tissues from both the rat and the rabbit as early as in 1940 [1]. About three decades later, it has been reported that 'pure' peroxisomes obtained from rat liver and kidney exhibited strong differences in their substrate specificity, notably with respect to glycolate and D,L-2-hydroxybutyrate [2]. This was shown to originate from the existence of two isozymic forms of L-2-HAOX which were further isolated and partially characterized [3]. The rat liver L-2-HAOX (isozyme A) preferentially oxidized short-chain aliphatic 2-hydroxyacids with maximal activity towards glycolate [4,5], whereas the kidney enzyme (isozyme B) catalysed the oxidation of long-chain aliphatic or aromatic 2-hydroxyacids at the highest rates [6,7].

Among mammalian hydroxyacid oxidases, rat kidney L-2-HAOX B [3,6,7], on the one hand, and L-2-HAOX A from rat [3], human [8,9] and pig [10] liver, on the other hand, have been purified and partially characterized. In vertebrates, the enzyme is believed to be involved in the metabolic production of oxalate through the successive oxidation of glycolate and glyoxylate [11], while in green plants it is one of the key enzymes in photorespiration [12]. Spinach glycolate oxidase is so far the sole FMN-dependent L-2-HAOX of the A-type for which the structure [13] and catalytic mechanism [14] have been well studied. The present work describes the purification and some molecular characteristics of chicken liver L-2-HAOX A in account of its involvement in the oxidation of supplemental methionine hydroxy analogue (2-hydroxy-4-methylthiobutanoic acid) and subsequent utilization for animal growth [15].

Correspondence address: A. Puigserver, C.N.R.S.-C.B.M., 31 chemin Joseph-Aiguier, 13402 Marseille Cedex 9, France

2. MATERIALS AND METHODS

2.1. *Chemicals*

Phenylmethanesulfonyl fluoride ($PheMeSO_2F$), horseradish peroxidase (Type II), and *o*-dianisidine dihydrochloride were from Sigma while glycolate was from Fluka. Standard proteins for electrophoresis calibration were obtained from Pharmacia. DEAE-cellulose (Whatman 52) came from Interchim, while AcA 34 and HA Ultrogel were from l'Industrie Biologique Française.

2.2. *Enzyme purification*

Frozen livers (400 g) from Arbor Acres strain adult chickens were thawed and rapidly homogenized [15] in 4 vols of 0.25 M sucrose, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $PheMeSO_2F$ and 10 mM Tris-HCl buffer, pH 8. L-2-HAOX activity from the peroxisomal fraction [2] was concentrated by ammonium sulfate precipitation (20–45% saturation), and the resulting precipitate dissolved in the minimal volume of 10 mM Tris-HCl, pH 8, containing 15 mM NaCl, 20 μM FMN and 1mM EDTA (buffer A). The solution was then dialysed against 500 vols of the latter buffer, applied to a column of DEAE-cellulose (3.5 × 20 cm) equilibrated with buffer A in the absence of FMN (buffer B), and the active fractions were eluted with a linear NaCl gradient (40–500 mM) and concentrated with a Diaflo cell fitted with an Amicon YM 100 membrane. After filtration through a column of AcA34 Ultrogel (2.5 × 200 cm), L-2-HAOX A activity was concentrated and further dialysed against a 10 mM phosphate buffer, pH 8, containing 1 mM EDTA, 1 mM $PheMeSO_2F$

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

and 20 μM FMN. Finally, the enzyme was adsorbed onto a column of HA Ultrogel (1.8×3 cm) equilibrated with the last buffer lacking FMN, eluted with a linear phosphate gradient (5–500 mM), readsorbed onto a second column of DEAE-cellulose (1.5×20 cm) equilibrated with buffer B and eluted with a linear NaCl gradient (15–300 mM). The enzyme purity was assessed by one-dimensional gel electrophoresis under usual sodium dodecyl sulfate denaturing conditions using 10% polyacrylamide slab gels [16].

2.3. *Antisera production*

Polyclonal antibody against highly purified chicken kidney L-2-HAOX A, from this laboratory, was prepared in the guinea-pig with 0.3 mg of antigen. The protein (0.1 mg) emulsified with complete Freund's adjuvant was first injected s.c. and a week later the same amount of protein emulsified with incomplete Freund's adjuvant was injected i.m. After an additional 10 day period, 0.05 mg antigen was injected s.c. and the next day again the same quantity was injected i.m. The antiserum was collected 10 days after the last injection.

2.4. *Western-blot analysis*

For immunodetection of proteins on nitrocellulose blots [17], the transferred proteins were first saturated with 10% bovine serum, and then incubated with a 1:500 dilution of the guinea-pig antiserum to L-2-HAOX A. The reacting antibodies were detected with a peroxidase-conjugated rabbit IgG to guinea pig IgG (heavy and light chains) in the presence of diaminobenzidine and H_2O_2.

2.5. *Amino acid analysis and automated Edman degradation*

The protein (100–200 pmol) was hydrolysed under vacuum with distilled 5.6 N HCl at 110°C, and its amino acid composition was subsequently determined with a Beckman Model 6300 autoanalyzer. The protein was reduced and carboxymethylated [18] prior to Edman degradation which was carried out on an Applied Biosystems sequencer Model 470 A, and phenylthiohydantoin (PTH) identification by means of an Applied PTH column (5 μm, 2.1×220 mm).

2.6. *Activity determination*

L-2-HAOX activity was determined by the procedure described in [19], with some modification, using a Kontron spectrophotometer (Uvikon 810) to monitor the increase in absorbance at 436 nm. The standard reaction mixture (1 ml) contained 0.23 mM *o*-dianisidine dihydrochloride, 6.25 mM L-2-hydroxy acid substrate, 2 IU horseradish peroxidase and 0.5 mM sodium azide in a 50 mM Tris-HCl buffer, pH 8.0. One unit of L-2-HAOX activity was defined as 1 nmol of L-2-hydroxy acid oxidized/min at 30°C using $\epsilon_{436} = 11.6 \times 10^{-3}$ M^{-1} cm^{-1} for oxidized *o*-dianisidine.

3. RESULTS AND DISCUSSION

3.1. *Enzyme purification and kinetic parameters*

The isolation of L-2-HAOX A from chicken liver consisted in ammonium sulfate precipitation of a peroxisomal fraction and subsequent chromatography on DEAE-cellulose, AcA 34- and HA Ultrogel, and again on DEAE-cellulose (Table I). When $(NH_4)_2SO_4$ precipitation was carried out at 45–65% saturation as in [3], a severe loss in L-2-HAOX A was observed (2% yield, not shown), and the final amount of enzyme did not exceed 0.06 mg starting from 200 g liver. Interestingly, the isolation procedure used in this study allowed us to obtain about 1.5 mg of highly purified enzyme from 400 g liver and the overall yield in enzyme activity was 27% while a 1400-fold purification of the protein was achieved. The Michaelis constant for the enzyme-catalysed oxidation of glycolate ($K_m = 0.10$ mM) was comparable to that determined with rat (0.50 mM [4]), man (0.33 mM [8]) and pig (0.42 mM [10]) liver isozyme A.

3.2. *Some molecular properties*

L-2-HAOX A isolated from chicken liver was homogeneous, as judged by SDS-polyacrylamide gel electrophoresis, and corresponded to a polypeptide chain of 39 kDa (not shown). In spite of slight differences, its amino acid composition (Table II) was comparable to those of L-2-hydroxylated oxidases from rat liver [3], pig liver [10] and spinach [13]. As in the case of mammals the enzyme contained several Cys residues but less Met and Ala contrastingly with the spinach enzyme. Its absorbance at 280 nm was rather low and in fair agreement with Trp content, and its molecular mass (37 760 Da), as derived from Table II, was consistent with that obtained by SDS-polyacrylamide gel electrophoresis. Since a value of 169 kDa was obtained under nondenaturing conditions (not shown), it was concluded that chicken liver L-2-HAOX A is composed of 4 apparently identical subunits as all the enzymes of the A-type so far isolated from mammals [3,7] and plants [13,20].

No N-terminal residue could be detected from the *S*-carboxymethylated protein, either manually by the dansyl-chloride method [21] or automatically with the aid of the Applied Biosystems sequencer. This indicated that the N-terminus of the polypeptide chain was blocked as that of spinach glycolate oxidase [13], but in contrast with that of rat kidney L-2-HAOX isozyme B

Table I

Purification of chicken liver L-2-hydroxyacid oxidase A

Purification step	Protein (mg)	Activity (units)	Specific activity (Units/mg)	Yield (%)	Purification (-fold)
Washed peroxisomes	5818	11637	1.5	100	1
Ammonium sulfate	3497	13989	4	120	3
DEAE-cellulose	48	7137	150	61	100
AcA 34 Ultrogel	9.5	4820	509	41	339
HA Ultrogel	3.7	3977	1075	34	717
DEAE-cellulose	1.5	3120	2080	27	1387

Table II

Amino acid composition of various L-2-hydroxyacid oxidases A

Residue	Chicken liver	Rat liver[a]	Pig liver[b]	Spinach[c]
Cys[d]	3.99 ± 0.07	4	5	1
Asx	29.40 ± 0.91	33	34	29
Thr	18.97 ± 0.48	19	16	21
Ser	18.87 ± 0.44	26	19	23
Glx	36.18 ± 0.91	44	35	31
Pro	21.05 ± 0.21	16	14	14
Gly	26.87 ± 0.88	30	26	27
Ala	26.16 ± 0.90	31	34	48
Val	25.67 ± 0.62	23	33	29
Met	8.07 ± 0.17	7	9	13
Ile	18.19 ± 0.54	ND	23	26
Leu	30.41 ± 1.05	30	36	31
Tyr	13.21 ± 0.38	11	10	9
Phe	14.83 ± 0.17	14	10	14
Lys	19.47 ± 0.49	25	25	18
His	8.09 ± 0.20	7	4	4
Arg	17.83 ± 0.59	15	22	26
Trp[e]	3.04 ± 0.11	5	6	5
Total number of residues per subunit	340	345	361	369
Total weight of residues	37760	37387	39967	38555

ND, not determined

[a,b,c] From references [3], [10] and [13], respectively

[d,e] Determined as *S*-carboxymethylated cysteine or as cysteic acid, and after hydrolysis with methanesulfonic acid, respectively

which was found to be Pro [7]. All these observations suggested that the presence of a N-terminal-blocked residue may be a feature proper to L-2-HAOX isozymes A.

The enzyme was quite stable for several weeks in Buffer A at 4°C, but could not be frozen at −20°C or −80°C. Its absorption spectrum in a 0.1 mM Tris-HCl buffer, pH 7.0, exhibited two peaks at 275 and 349 nm, the former being characteristic of the flavin chromophore.

3.3. *Immunochemical properties*

An antiserum was raised in the guinea-pig against homogeneous chicken kidney L-2-HAOX A; we were also able to purify (524-fold, specific activity: 1900 nmol glycolate oxidized/min/mg protein). Fig. 1 shows that when the peroxisomal fractions obtained from both chicken and rat liver and kidney were first subjected to SDS-polyacrylamide gel electrophoresis and further transferred to nitrocellulose prior to immunoblotting, the polyclonal antibody recognized similarly a 37 kDa polypeptide chain whatever the fraction may be. Thus the antibody raised against chicken kidney L-2-HAOX A cross-reacted with L-2-HAOX

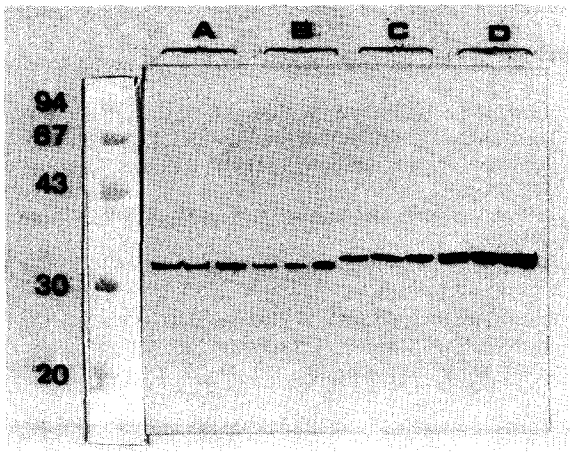

Fig. 1. Immunoblotting of the 'washed' peroxisomal fractions from chicken and rat tissues. Proteins were transferred from the 10% acrylamide gel to a nitrocellulose sheet and revealed using the polyclonal antibodies raised against chicken kidney. From left to right: molecular weight markers stained with Ponceau red, chicken liver enzyme (lanes A: 10, 20 and 50 μg), chicken kidney enzyme (lanes B: 5, 10 and 25 μg), rat liver enzyme (lanes C: 10, 20 and 50 μg) and rat kidney enzyme (lanes D: 7, 15 and 35 μg).

isozyme B from rat kidney. However, when Ouchterlony double immunodiffusion experiments were carried out (not shown), precipitin lines were observed with the isozyme A from both species but not with rat kidney isozyme B. This result is consistent with previous findings [22] where no cross-reactivity between rat liver L-2-HAOX A or kidney L-2-HAOX B and the alternate antibody could be detected by enzyme activity titration as well as by Ouchterlony's test. Immunological identity between the isozymes A and B from rat tissues has nevertheless been pointed out, suggesting that both forms could be derived from recently duplicated genes [23]. The existence of some structural identity between isozymes A and B in the higher organisms is reasonable in view of the present results since our polyclonal antibody was able to recognize common epitopes in both denaturated isozymes but not in their native forms.

Acknowledgements: We are grateful to Mrs A. Guidoni and Mrs M. Woudstra for amino acid analyses, Mr J. Bonicel for sequence determination and Dr A. De Caro for pertinent advice during immunochemical experiments.

REFERENCES

[1] Dohan, J.S. (1940) J. Biol. Chem. 135, 793–794.
[2] De Duve, C. and Baudhuin, P. (1966) Physiol. Rev. 46, 323–357.
[3] Duley, J.A. and Holmes, R.S. (1976) Eur. J. Biochem. 63, 163–173.
[4] Nakano, M., Ushishima, Y., Saga, M., Tsutsumi, Y. and Asami, H. (1968) Biochim. Biophys. Acta 167, 9–22.
[5] MacGroarty, E., Hsieh, B., Wied, D.M., Gee, R. and Tolbert, N.E. (1974) Arch. Biochem. Biophys. 161, 194–210.
[6] Cromartie, T.H. and Walsh, C.T. (1975) Biochemistry 14, 2588–2596.

[7] Urban, P., Chirat, I. and Lederer, F. (1988) Biochemistry 27, 7365–7371.
[8] Fry, D.W. and Richardson, K.E. (1979) Biochim. Biophys. Acta 568, 135–144.
[9] Schwam, H., Michelson, R., Randall, W.C., Sondey, J.M. and Hirschmann, R. (1979) Biochemistry 18, 2828–2833.
[10] Schuman, M. and Massey, V. (1971) Biochim. Biophys. Acta 227, 500–520.
[11] Richardson, K.E. and Tolbert, N.E. (1961) J. Biol. Chem. 236, 1280–1285.
[12] Lindqvist, Y. and Brandén, C.I. (1985) Proc. Natl. Acad. Sci. USA 82, 6855–6859.
[13] Cederlund, E., Lindqvist, Y., Söderlund, G., Brandén, C.I. and Jornväll, H. (1988) Eur. J. Biochem. 173, 523–530.
[14] Lindqvist, Y. and Brandén, C.I. (1989) J. Biol. Chem. 246, 3624–3628.
[15] Dupuis, L., Saunderson, C.L., Puigserver, A. and Brachet, P. (1989) Br. J. Nutr. 62, 63–75.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
[18] Charles, M., Erlanson, C., Bianchetta, J., Joffre, J., Guidoni, A.A. and Rovery, M. (1974) Biochim. Biophys. Acta 359, 186–197.
[19] Duley, J.A. and Holmes, R.S. (1975) Anal. Biochem. 69, 164–169.
[20] Behrends, W., Rausch, U., Löffler, H.-G. and Kindl, H. (1982) Planta 156, 566–571.
[21] Bianchetta, J.D., Bidaud, J., Guidoni, A.A., Bonicel, J.J. and Rovery, M. (1979) Eur. J. Biochem. 97, 395–405.
[22] Yokota, S., Ichikawa, K. and Hashimoto, T. (1985) Histochemistry 82, 25–32.
[23] Duley, J. and Holmes, R.S. (1977) Int. J. Biochem. 8, 127–130.