Volume 336, number 2, 272–274 FEBS 13435 December 1993

Retinal oxidase is identical to aldehyde oxidase

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Received 24 October 1993

Retinal oxidase (EC 1.2.3.11) and aldehyde oxidase (EC 1.2.3.1) were compared with respect to their enzymatic and physicochemical properties. It was found that the molecular weights, subunits, optical spectra, chemical and immunological properties, cellular localization and substrate specificities of the two oxidases are identical on almost all points. The physiological function of aldehyde oxidase appears to be the synthesis of retinoic acid from retinal.

Retinal oxidase; Aldehyde oxidase; Retinoic acid synthase; Flavoenzyme; Oxidoreductase

1. INTRODUCTION

Retinoic acid is a lipoactivator and has multiple functions, such as the formation of a limb bud and a rhombencephalon, growth control of epithelial tissues, cellular differentiation and morphogenesis [1,2]. Recently, we reported that the retinoic acid in rabbit liver is synthesized by retinal oxidase (EC 1.2.3.11) [3] in the cytosol and a cytochrome P-450-linked monooxygenase system [4,5] of microsomes.

In this study, we investigated and compared the enzymatic and physicochemical properties of retinal oxidase (EC 1.2.3.11) and aldehyde oxidase (EC 1.2.3.1). It was found that retinal oxidase has entirely the same properties as aldehyde oxidase, and so we concluded that retinal oxidase is identical to aldehyde oxidase.

2. MATERIALS AND METHODS

2.1. *Materials*

Healthy male rabbits weighing 2.0 to 2.5 kg (Japanese white rabbits, 6 months old) were fed on a standard commercial laboratory diet with water ad libitum.

Rabbit liver cytosol was prepared from rabbit livers by the method of Siekevitz [6]. To obtain the postmicrosomal fraction of the rabbit liver homogenate, the postmitochondrial fraction was centrifuged at 105,000 × *g* for 2 h. The resulting supernatant was used as the cytosol.

2.2. *Purification of retinal oxidase and aldehyde oxidase*

Retinal oxidase and aldehyde oxidase were purified from rabbit liver cytosol by the methods of Tsujita et al. [3] and Stell et al. [7], respectively. The oxidases each gave an electrophoretically single protein band on SDS-PAGE, and a single absorption peak at 280 nm on HPLC with a TSK-gel G3000swXL column (7.5 cm × cm).

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Abbreviations: NMNA, *N*-methylnicotinamide; NBT, Nitro blue tetrazolium; DCIP, dichlorophenolindophenol.

2.3. *Enzyme assays for retinal oxidase and aldehyde oxidase*

The activity of retinal oxidase was measured with various retinoids and xenobiotics by HPLC under dark conditions by the method of Tsujita et al. [3]. The activity of aldehyde oxidase was measured with the substrates for retinal oxidase by the method of Felsted et al. [8].

The activities of cytochrome *c* reductase and diaphorases with DCIP, ferricyanide or NBT were measured spectrophotometrically in 50 mM potassium phosphate buffer, pH 7.8, at 25°C by the methods of Krenitsky et al. [9] and Branzoli et al. [10], respectively. The cytochrome *c* reductase activity was also studied aerobically in the absence and presence of 1 μM superoxide dismutase.

2.4. *Kinetic parameters*

Kinetic parameters for various substrates of retinal oxidase and aldehyde oxidase were determined by the method of Krenitsky et al. [9].

2.5. *Analyses of contents of flavins, metals, labile sulfurs and amino acid residues*

FAD and FMN were analyzed by the method of Felsted et al. [8]. Metal contents were determined with a Hitachi atomic absorption spectrophotometer, type Z-900 (Tokyo, Japan). The labile sulfur content was determined by the method of Fogo and Popowsky [11]. The amino acid contents were determined by the method of Moore and Stein [12], using a PICO-TAG™ work station (Millipore, Milford, MA).

2.6. *Chemicals*

All-trans retinal, *9-cis* retinal, *13-cis* retinal, *all-trans* retinoic acid, *N*-methylnicotinamide (NMNA), purine, 2-hydoxypyrimidine, hypoxanthine, xanthine, cytochrome c and superoxide dismutase (from bovine liver) were purchased from Sigma Chemical Co. (St. Louis, MO). Ferricyanide, Nitro blue tetrazolium (NBT) and dichlorophenol- indophenol (DCIP) were obtained from Wako Chemical Co. (Osaka, Japan). A standard protein kit for gel filtration was obtained from the Bio-Rad Chemical Division (Richmond, CA).

All other reagents were of the best grade available from commercial sources.

3. RESULTS

Retinal oxidase and aldehyde oxidase were purified electrophoretically as single proteins from rabbit liver cytosol. The characteristic properties of the two retinal

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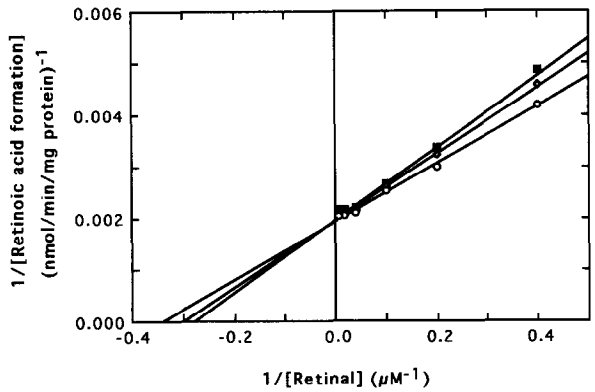

Fig. 1. Effect of NMNA on the activity of retinal oxidase. The activity of retinal oxidase was assayed in the presence of 1.0 and 0.5 mM NMNA. ○, without NMNA; ◇, 0.5 mM NMNA; ■, 1 mM NMNA.

and aldehyde oxidases were compared with respect to molecular weights, subunits, optical spectra, and flavin and metal contents. The results are summarized in Table I. Both oxidases were optically typical molybdoflavoenzymes. The molecular weight of retinal oxidase was estimated to be 270 kDa by HPLC and that of aldehyde oxidase was the same. The molecules of the two oxidases both comprised homodimers of 135 kDa.

The physical properties of the two oxidases are consistent with each other. The contents of molybdenum, iron, FAD and labile sulfur were qualitatively and quantitatively the same in both oxidases. Accordingly, retinal oxidase and aldehyde oxidase could not be distinguished on the basis of the chemical properties.

The substrate specificity of retinal oxidase was examined with respect to various substrates, such as retinals, pyridines, pyrimidines and purines, and compared with that of aldehyde oxidase. The results are summarized in Table II. As shown in this table, the substrate specificity of aldehyde oxidase confirmed that previously reported by Krenitsky et al. [9]. The substrate specificities of both oxidases are also expressed as the relative activities and the apparent K_m values for substrates. These activities of the two oxidases were mutually similar.

The activities of cytochrome *c* reductase and diaphorases of both oxidases with NMNA were examined aerobically and anaerobically in the presence and absence of 1 μM superoxide dismutase. The diaphorase activities were measured by using ferricyanide, DCIP or NBT. The results are summarized in Table III. The activities of cytochrome *c* reductase of retinal oxidase and aldehyde oxidase could not be detected in the presence of 1 μM superoxide dismutase. The activities were expected to be caused by superoxide produced via oxidases. However, the diaphorase activities of retinal oxidase and aldehyde oxidase were not affected by the superoxide dismutase. This table demonstrates that the K_m values and turnover numbers of retinal oxidase are identical to those of aldehyde oxidase. Furthermore, it was found that the activity of retinal oxidase was inhibited competitively by substrates such as NMNA of aldehyde oxidase. The K_i value for NMNA of retinal oxidase was 2.1 mM (Fig. 1). Anti-retinal oxidase IgG inhibited the activity of aldehyde oxidase and that of

Table I

Chemical compositions and molecular weights of retinal oxidase and aldehyde oxidase

Physical constant (per one molecule)	Retinal oxidase	Aldehyde oxidase	
		This study	References
Molecular weight	270 kDa	270 kDa	270–280 kDa[a]
Subunit	2 at 135 kDa	2 at 135 kDa	–
FAD	2.0	2.0	1.8[b]
Iron	8.24	8.3	8.2[b]
Molybdenum	2.01	2.0	2.0[b]
Labile sulfur	7.86	–	–

[a] Nelson and Handler [13]
[b] Rajagopalan et al. [14]

Table II

Michaelis constants for substrates of retinal oxidase and aldehyde oxidase, and their relative activities

Substrate	Retinal oxidase	Aldehyde oxidase	
	K_m (M)		
all-trans retinal	8×10^{-6}	7.4×10^{-6}	
9-cis retinal	1.3×10^{-5}	1.5×10^{-5}	
13-cis retinal	9×10^{-4}	10×10^{-4}	
NMNA	0.7×10^{-3}	0.9×10^{-3}	(1×10^{-3})
Purine	2.2×10^{-4}	2.0×10^{-4}	(3×10^{-4})
Hypoxanthine	1.4×10^{-3}	2.1×10^{-3}	(2×10^{-3})
Xanthine	1.7×10^{-3}	1.3×10^{-3}	(2×10^{-3})
Substrate	Retinal oxidase	Aldehyde oxidase	
	V_{max} (nmol/min/mg protein)		
all-trans retinal	496	441	
9-cis retinal	57	60	
13-cis retinal	105	98	
	Relative activity (%)		
Purine	100 (%)	100 (%)	100 (%)
NMNA	60	45	(34)
Xanthine	1.9	1	(< 1)
Hypoxanthine	26	22	(3)
Allopurinol	12	13	(16)
2-Hydroxypyrimidine	129	141	(280)

Ferricyanide was used as the electron acceptor under the conditions described by Krenitsky et al. [9] except for the activity of retinal oxidase. The activities of retinal oxidase with retinals were measured as the synthesis of retinoic acid. The values in parentheses are quoted from Krenitsky et al. [9]. Relative values is expressed as a percentage of the purine oxidase activities of the retinal oxidase and aldehyde oxidase.

Table III

Michaelis constants and turnover numbers of retinal oxidase and aldehyde oxidase with respect to some electron acceptors, and effects of superoxide dismutase on these activities

Acceptor	Retinal oxidase		Aldehyde oxidase			Effects of superoxide dismutase at 10^{-6} M (turnover number, min^{-1})	
	K_m value	Turnover number (min^{-1})	K_m value		Turnover number (min^{-1})	Retinal oxidase	Aldehyde oxidase
Cytochrome *c*	0.85 μM[b]		0.92 μM[b]	(0.8 μM)[b]			
	1.56 mM[a]	26.8	1.9 mM[a]	(1.37 mM)[a]	32 (32)	0.0	0.0 (complete inhibition)
Ferricyanide	0.49 mM[a]	118	0.38 mM[a]	(0.32 mM)[a]	135 (230)	118	135 (none)
DCIP	0.21 mM[a]	108.4	0.18 mM[a]	(0.22 mM)[a]	121 (200)	108.4	121 (none)
NBT	86.0 μM[c]		75 μM[c]	(70 μM)[c]		68.7	72 (none)
	0.22 mM[a]	68.7	0.29 mM[a]		72 (74)		

The activities of cytochrome *c* reductase and diaphorases were assayed in the presence of an electron acceptor with NMNA as a substrate. The values in parentheses are quoted from Branzoli and Massey [10].

[a] K_m for NMNA.
[b] K_m for cytochrome *c*.
[c] K_m for NBT.

retinal oxidase to the same degree (data not shown). This result indicated that the retinal oxidase is immunochemically similar to aldehyde oxidase.

4. DISCUSSION

Retinal oxidase (EC 1.2.3.11) and aldehyde oxidase (EC 1.2.3.1) were purified from rabbit liver cytosol, and their physicochemical, enzymatic and immunochemical properties were compared. It was found that retinal oxidase was physicochemically, enzymatically and immunologically identical to aldehyde oxidase. The results of chemical analyses of the retinal oxidase were identical to those in the case of the aldehyde oxidase, which was reported previously by other workers [7,13,14]. On comparison of their substrate specificifties, the K_m values of the two oxidases for retinals were consistent. The cytochrome *c* reductase activities of both oxidases, like that of xanthine oxidase (EC 1.1.3.22) [15,16], were inhibited completely by superoxide dismutase. However, we previously reported that xanthine oxidase was completely different from retinal oxidase and aldehyde oxidase [3]. The activity of retinal oxidase was competitively inhibited by NMNA, a substrate of aldehyde oxidase. These results supported the hypothesis that retinal oxidase is identical to aldehyde oxidase.

Although the physiological function of aldehyde oxidase has not been established yet, a major role of aldehyde oxidase appears to be the synthesis of retinoic acid from retinal.

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