Ornithine decarboxylase activity in embryos depends on temperature of development rather than on the stage of development

Molecular adaptation to temperature changes in poikilothermic animals

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The activity of ornithine decarboxylase (ODC) (the key enzyme of polyamine synthesis) in different poikilothermic animals depends on the temperatures at which they were kept just before the enzyme assay. With an increase in temperature (within physiological limits) ODC activity rises 5–25-fold within several hours. With a decrease in temperature it falls at the same rate. This effect, studied on loach (*Misgurnus fossilis*) embryos in detail, was also shown for embryos, larvae and some adult tissues of many species. It is not, however, observed in homoiothermic animals (chick embryos and mammalian cells), nor in bacteria and plants. Changes in polyamine concentrations follow those in ODC activity, but more slowly and to a lesser extent. It is assumed that modulation of ODC activity changes as a result of its synthesis and degradation. We suggest that the temperature-dependence of ODC activity is a mechanism of adaptation which maintains the optimal cellular concentration of polyamines for each temperature.

It is commonly assumed that, within the physiological temperature limits, metabolic processes of poikilothermic animals are balanced and that there are no special mechanisms of rapid biochemical adaptation to temperature changes. Only in animals kept at high or low temperatures for long periods (days, weeks) are moderate differences in activities of some enzymes observed. These slow changes are usually of a compensatory character, i.e. the enzyme activity is decreased at high temperatures, and vice versa (Hochachka & Somero, 1973; Prosser, 1973; Schmidt-Nielsen, 1979). In distinction to these facts, the present report shows that ODC activity rises quickly and significantly after the temperature of development is increased, and falls rapidly after the temperature is decreased. We observed this unusual effect in embryos and larvae of fish and many poikilothermic species.

ODC converts ornithine into putrescine by removal of CO₂. This is the key reaction in the

Abbreviation used: ODC, ornithine decarboxylase (EC 4.1.1.17).

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formation of the polyamines spermidine and spermine (Tabor & Tabor, 1976; Heby, 1981). The ODC activity changes quickly, depending on the functional state of the cell: it rises when cells begin proliferation or differentiation (Manen & Russell, 1973; Lundquist *et al.*, 1983). A similar increase occurs under the action of hormones or carcinogens (for reviews, see Heby, 1981; Abraham & Pihl, 1981; Goyns, 1982). The polyamines are able to bind negatively charged phosphate groups of nucleic acids and to form links between them, owing to their positively charged amino groups (Munro & Bell, 1973; Becker *et al.*, 1979; Burton *et al.*, 1981; Igarashi *et al.*, 1982). These links stabilize the structure of nucleic acids which may be necessary for their functions (Heby, 1981), e.g. replication, transcription and translation, and probably for ensuring the fidelity of the template processes (Abraham & Pihl, 1981). The polyamines are also reported to bind the proteins (Kuehn *et al.*, 1979; Canellakis *et al.*, 1980; Haddox & Russell, 1981). On the basis of the present work we propose that rapid changes of ODC activity are the manifestation of 'molecular adaptation' to temperature changes. It seems that for each temperature there is an optimal polyamine concentration.

Materials and methods

Animals

The mature eggs of loach (*Misgurnus fossilis*) were obtained through gonadotropin stimulation (Choriogonin; G. Richter, Budapest, Hungary). Eggs were fertilized artificially and embryos were raised at different temperatures. The number of the morphological stage is designated as the number of hours of development at 21°C (Neyfakh, 1959). Temperature, stages of development and time of incubation before ODC assay for other species are listed in Table 1.

Materials

L-[1-¹⁴C]Ornithine was obtained from Amersham International. L-Ornithine, dithiothreitol and cycloheximide were from Sigma, and actinomycin D was from Serva.

Assay of ODC activity

The loach embryos reaching the same morphological stages at different temperatures (and accordingly for different time periods) were homogenized (Teflon/glass, 1 min, 4°C) in 2 vol. of buffer [0.1 M-Tris/HCl (pH 7.6)/5 mM-dithiothreitol/0.5 mM-EDTA] and centrifuged (15 000 g, 10 min). The same method of homogenization was used for other tissues. For determination of ODC activity, 0.3 ml of the supernatant was put into a weighing bottle (30 mm × 30 mm) with a tight glass stopper, and 0.2 ml of a solution (Jänne & Williams-Ashman, 1971) containing 0.1 M-Tris/HCl, pH 7.6, 5 mM-dithiothreitol and 0.1 mM-pyridoxal 5-phosphate, 1 mM-L-ornithine and 0.05 µCi of L-[1-¹⁴C]ornithine (60 mCi/mmol) was added. For CO₂ absorption, Whatman 3MM filter paper moistened with 50 µl of 3 M-KOH/0.4 M-Ba(OH)₂ was fixed to the lower side of the stopper. Incubations were performed at 37°C for 60 min. The reaction was stopped by adding 0.3 ml of 50% (w/v) trichloroacetic acid, and then incubation was continued for 60 min to complete CO₂ absorption. Filters were dried and counted for radioactivity in toluene-based scintillator (SL-30; Intertechnique). In controls 0.3 ml of buffer was added instead of homogenate. The reaction rate was linear with time up to 2 h under these conditions. The protein content was determined by the Lowry method in samples of the supernatant. ODC activity was expressed in nmol of CO₂ released/h per mg of protein (Jänne & Williams-Ashman, 1971).

Estimate of ODC activity in vivo

The embryos were raised up to stage 22 (first somites) at 21°C ('warm') or at 11°C ('cold'), isolated from the yolk (Kostomarov & Neyfakh, 1964) and placed (200–250 embryos) into weighing bottles (30 mm × 30 mm) with tight glass stoppers,

containing 0.5 ml of double Holtfreter (1931) solution. To each bottle 0.1 µCi of L-[1-¹⁴C]-ornithine (60 mCi/mmol) was added. A Whatman 3MM filter paper moistened with 50 µl of 3 M-KOH/0.4 M-Ba(OH)₂ was fixed to the lower side of the stopper.

Just after addition of [¹⁴C]ornithine and after 30 min of incubation at 21°C, samples (20 µl) of medium were taken from each weighing bottle and placed on separate filters. After incubation (30 min, 21°C), 0.3 ml of 50% (w/v) trichloroacetic acid was added into the bottles, and then the incubation was prolonged for 60 min at 37°C to complete CO₂ absorption. All three filters from each bottle ([¹⁴C]-ornithine in medium at the beginning and at the end of incubation, and ¹⁴CO₂ released) were dried and counted for radioactivity in toluene-based scintillator (SL-30; Intertechnique). The total quantity of [¹⁴C]ornithine absorbed by the 'warm' and 'cold' embryos during 30 min incubation at the same temperature (21°C) and ¹⁴CO₂ released were calculated. ODC activity *in vivo* is expressed as percentage of [¹⁴C]ornithine decarboxylated.

Determination of polyamines

Each batch of fertilized loach eggs was divided into two portions, which were incubated at 21°C ('warm') or 12°C ('cold'). After they had reached defined morphological stages, they were isolated from the yolk (Kostomarov & Neyfakh, 1964), homogenized and diluted to give equal concentrations of protein. The protein was then precipitated by sulphosalicylate, and supernatants were used for determination of polyamines. Polyamines were separated and assayed on an amino acid analyser by Dr. L. Baratova. Details of these experiments and methods are described by Baratova *et al.* (1984). The quantity of polyamines is expressed per mg of protein of the initial homogenate.

Micro-injection of actinomycin D

A 20 nl portion of a saturated solution of actinomycin D in 10% (v/v) ethanol (approx. 500 µg/ml) was micro-injected into each loach egg at stage 2–4 blastomere. For the injection we used a high-pressure syringe connected to a glass needle (diameter 20 µm) (Korzh, 1981). The concentration of the drug once in the eggs was approx. 15 µg/ml, which inhibited RNA synthesis by approx. 90% and arrested the development in the late-blastula stage.

Results

Dependence of ODC activity on the temperature of development

ODC activity was assayed in extracts obtained from the embryos of loach (*Misgurnus fossilis*,

Fig. 1. ODC activity in loach embryos developing at different temperatures

The temperatures of development are shown on the curves. ODC activity was always assayed at 37°C. Note that the same morphological stages were reached at different temperatures over different periods of time.

Teleostei). The embryos were incubated at different temperatures (8–21°C) until they reached the defined morphological stages of development (Fig. 1). The same morphological stages were reached at 17°, 14°, 11° and 8°C respectively, 1.5, 2.1, 3.3 and 6.7 times slower than at 21°C. In all extracts the ODC activity was always assayed at a constant temperature of 37°C. (This temperature was chosen since control experiments at 25°C gave essentially the same results, although the activity was almost three times less. Besides, the loach ODC was inactivated by 50% for 60 min even at 48–49°C. These results are not presented here.)

The activity increased in the early embryos incubated after fertilization at 21°, 17° and 14°C, whereas in the embryos incubated at 11° and 8°C it decreased (Fig. 1). This difference between the ‘warm’ and ‘cold’ loach embryos was increased when the yolk was removed before homogenization (Table 1). ODC activity is probably the first known example in which an enzyme activity depends primarily not on the stage of development but rather on the temperature at which the development has taken place.

Similar results were obtained for embryos, larvae or adults of other species of poikilothermic animals developed or incubated at the high and low temperatures within the physiological range (Table 1). In the experiments ODC activity at a given stage

Table 1. Activity of ODC in representatives of various species, preincubated at different temperatures
Embryos or adult organisms of various species were preincubated for various times at different temperatures within physiological limits. ODC activity was assayed at 37°C and expressed in nmol of released CO₂/h per mg of protein. The ratio of ODC activities after preincubation at maximum and minimum temperatures is presented (column 5). All experiments were repeated at least two or three times with similar results (data from one experiment are presented).

	Time	Temperature (°C)	ODC activity	Ratio
Bacteria				
<i>E. coli</i> K12	20 h	21	176	} 1.0
		37	177	
Plants				
Maize, <i>Zea mays</i>				
Roots	4 days	11	6.4	} 1.2
		25	7.8	
Sprouts	4 days	11	3.3	} 0.9
		25	3.0	
Coelenterates				
<i>Hydra olygactis</i>	24 h	8	1.0	} 1.7
		14	1.1	
		22	1.2	
		25	1.7	
Flatworms				
Planarian, <i>Dugesia lugumbris</i>	24 h	18	0.041	} 2.5
		26	0.099	
Insects				
<i>Drosophila melanogaster</i> , 3rd instar	12 h	12	0.06	} 2.5
		25	0.15	

Table 1—continued

Fishes				
Sturgeon, <i>Asipenser stellatus</i>				
Gastrula	6 h	12	2.4	} 4.7
		18	11.0	
		10	1.6	
Larva	24 h	16	3.2	} 3.7
		22	6.0	
		Salmon, <i>Salmo ischan</i>		
Stage 24 (eye pigmentation)	20 h	1	0.053	} 20.2
		12	1.07	
Loach, <i>Misgurnus fossilis</i>				
Stage 6 (early blastula)	6 h	8	0.02	} 41.0
		11	0.10	
		14	0.32	
		17	0.63	
		21	0.82	
Stage 22 (first somites) intact embryos	12 h	11	0.17	} 12.3
		21	2.1	
embryos without yolk	12 h	11	1.1	} 19.1
		21	21.1	
Amphibians				
Toad, <i>Bufo bufo</i>				
Late gastrula	12 h	8	0.20	} 9.0
		11	0.54	
		17	0.62	
		25	1.79	
		28	1.82	
African clawed toad, <i>Xenopus laevis</i>				
Tadpoles	24 h	18	0.27	} 2.4
		26	0.66	
Adult organs				
Liver	24 h	11	0.027	} 5.8
		18	0.097	
		30	0.154	
Intestine	24 h	11	0.23	} 1.4
		18	0.27	
		30	0.32	
Kidney	24 h	11	0.10	} 5.9
		18	0.53	
		30	0.59	
Reptiles				
Grass snake, <i>Natrix natrix</i>				
3-week embryos	48 h	11	0.07	} 13.7
		17	0.21	
		25	0.86	
		29	0.96	
Birds				
Chick embryos 6 days	4 h	31	5.4	} 1.1
		34	7.2	
		37	5.9	
		(40.2)	(1.6)	
Mammals				
Chinese-hamster fibroblasts (cell culture)	4 h	31	1.06	} 0.36
		34	0.61	
		37	0.39	
		(40.0)	(0.13)	

Table 2. Activity of ODC *in vivo* in 'warm' and 'cold' loach embryos

Loach embryos developed up to Stage 22 (first somites) at 21°C ('warm') or at 11°C ('cold') were isolated from the yolk and incubated for 30 min at 21°C in salt solution with [^{14}C]ornithine. ODC activity was expressed as the percentage of decarboxylated [^{14}C]ornithine absorbed by the embryos (see the Materials and methods section). Data from four independent experiments are presented.

	Quantity of [^{14}C]- ornithine absorbed by embryos in 30 min (d.p.m.)	Quantity of $^{14}\text{CO}_2$ released by embryos in 30 min (d.p.m.)	ODC activity (%)
'Warm' embryos	37 200	9650	26
	40 810	8630	21
	36 240	8760	24
	41 200	8210	20
			Mean 22.7
'Cold' embryos	64 060	3280	5.1
	78 820	2940	3.7
	59 200	3400	5.7
	83 670	2740	3.2
			Mean 4.4

of development was positively correlated with the temperature for representative coelenterates, flatworms, insects, fish (sturgeon, salmon), amphibians and reptiles. Differences in ODC activity were also found in some organs of adult *Xenopus laevis* maintained at different temperatures for 24 h. But no such differences were found in bacteria (*Escherichia coli*) and in plants (maize), where ODC is not the key enzyme for polyamine biosynthesis and other pathways are predominant (Heby, 1981). Although the conditions of the experiment (species, time of preincubation, temperatures) were chosen arbitrarily (in distinction to the loach embryos, which were studied carefully), it can be seen that in the more primitive organisms and in adult animals the dependence of ODC activity on temperature is less. It is very interesting that such differences could not be found also in homoiothermic animals, e.g. birds (chick embryo) and mammals (Chinese-hamster fibroblasts).

To ascertain whether the ODC activity *in vivo* depends on the temperature of preceding development, the release of $^{14}\text{CO}_2$ from [^{14}C]ornithine absorbed by loach embryos was determined. The embryos were raised at 21° or 14°C, but the ODC activity was always measured at 21°C. The 'warm' embryos produced several times more $^{14}\text{CO}_2$ than the 'cold' ones at the same stage of development (Table 2). This confirms the results *in vitro*. These results can, however, be overestimated with respect to [^{14}C]ornithine oxidized without decarboxylation to putrescine (Murphy & Brosnan, 1976). The difference between the 'cold' and the 'warm' embryos in this experiment *in vivo* might have been somewhat underestimated, because any possible

increase in ODC activity of the 'cold' embryos during the 30 min assay at 21°C was ignored.

In a small series of experiments other enzymes of polyamine biosynthesis (putrescine- and spermidine-dependent *S*-adenosyl-L-methionine decarboxylases) were examined; their activities were also elevated at higher temperatures. However, the difference ranged from 2- to 2.5-fold and was not as pronounced as that in ODC activity.

Rate of alterations in ODC activity after shifts in temperature of development

The loach embryos were transferred from 11° to 21°C and vice versa, and ODC activity in the extracts was determined at different time intervals after transfer (Fig. 2). The changes in the enzyme activity were detectable as early as 10 min after the transfer. ODC activity continued to increase or decrease, depending on the temperature changes involved in the experiment. In 2–3 h it adjusted to that characteristic of the new temperature.

In the presence of cycloheximide (20–100 µg/ml), no increase in ODC activity took place after the embryos were transferred to a higher temperature (Fig. 3a). However, if 'warm' embryos were transferred to the lower temperature, ODC activity decreased almost independently of whether the protein-synthesis inhibitor was present in the embryos or not (Fig. 3b). Moreover, cycloheximide alone produced the same decrease in the ODC activity at 21°C as the transfer of the embryos to 11°C. These experiments show that protein synthesis is required both for the maintenance of ODC activity at the high value at 21°C and for the increase of the enzyme activity upon accommo-

dation to this temperature. This is in good agreement with the fact that ODC has the shortest half-life at present known of any enzyme (in mammals approx. 10min) (Heby, 1981; Abraham & Pihl, 1981). Evidently the temperature-dependent increase in ODC activity can be affected by intensification of the synthesis of either the enzyme itself or its protein activator. The decrease in ODC activity after cooling or in the presence of protein-synthesis

inhibitor could be due to the degradation of such a short-lived enzyme. The half-life for the loach ODC is approx. 1–1.5h *in vivo* (Fig. 3). In distinction to cycloheximide, actinomycin D did not penetrate into loach eggs and therefore was micro-injected 1.5h after fertilization (2–4-blastomere stage). We found that actinomycin D neither influences the change in ODC activity during the first hours of development (i.e. activity up at 21°C and down at 11°C) nor inhibits the increase in ODC activity after the embryos are transferred from 11° to 21°C in the blastula stage (results not shown).

Fig. 2. Rate of ODC-activity changes in loach embryos after the temperature shift

Loach embryos were developed to the late-blastula stage at 11°C (24h), and at zero time were transferred to 21°C (○, 11°→21°). Another group of embryos were developed to the mid-gastrula stage at 21°C (12h), and at zero time were transferred to 11°C (●, 21°→11°).

Temperature-dependent changes in polyamine content

The contents of putrescine, spermidine and spermine in the loach embryos were assayed in 'warm' and in 'cold' embryos. At the early embryonic stages of development at different temperatures (12° and 21°C) there was relatively little difference in content of polyamines. However, these differences increased with embryogenesis (Table 3). Thus polyamine content is changed at a slower rate and to a lesser extent than is ODC activity.

Discussion

On the basis of the present data (Fig. 3) it is proposed that, although the increase in ODC activity at a high temperature (but within the physiological range) occurs by means of increased enzyme synthesis, the decrease is due both to the cessation of synthesis and to a high rate of degradation. These temperature-dependent modu-

Fig. 3. Effect of cycloheximide on ODC activity on temperature changes

(a) The loach embryos developed to the late-gastrula stage at 11°C. Some of them were transferred to 21°C at zero time (○, ●, 11°→21°). (b) Embryos developed to the mid-gastrula stage at 21°C. Some of them were transferred to 11°C at zero time (△, ●, 21°→11°). Before the change of incubation temperature, some embryos of all variants were placed into cycloheximide solution (100 µg/ml) (a and b: □, ●).

Table 3. Content of polyamines in loach embryos developing at 12°C ('cold') and 21°C ('warm')

Embryos were developed up to stage 9 (late blastula) for 9 h at 21°C or for 27 h at 12°C, and to stage 55 (hatching) for 55 h at 21°C or for 170 h at 12°C. The content of polyamines is expressed in nmol/mg of protein of the initial homogenate (see the Materials and methods section). The ratios of values for 'warm' embryos to those of the 'cold' ones are shown in parentheses.

	Content (nmol/mg)	
	Stage 9	Stage 55
Putrescine		
'Warm'	25	60
'Cold'	21 (1.2)	35 (1.7)
Spermidine		
'Warm'	12	25
'Cold'	9 (1.3)	12 (2.2)
Spermine		
'Warm'	1.7	1.7
'Cold'	1.1 (1.5)	0.8 (2.2)

lations of ODC activity are not sensitive to actinomycin and can be observed at all stages of development, including early fish embryogenesis (up to the gastrula), when practically no transcription takes place (Neyfakh, 1971). It can therefore be concluded that the regulation of ODC activity may occur at the translational level, e.g. on the templates stored during oogenesis. The fluctuations in ODC activity may occur by means of temperature-dependent selective translation of enzyme molecules. However, these fluctuations also may be mediated by the temperature-dependent changes in the ratio of rate constants for synthesis and degradation (see Schimke & Doyle, 1970; Tabor & Tabor, 1976). Measurement of these constants for ODC is difficult.

It is not obvious that the morphologically similar embryos which reached the same stage at different temperatures are identical in all respects (aside from their differences in ODC activity and polyamine content). Firstly, it may be important to know the content of nucleic acids, because the polyamines can vary co-ordinately with them. It is known, however, that the rate of morphological development is strongly proportional to the rate of cleavage divisions within the limits of physiological temperatures (Dettlaff & Dettlaff, 1961). Therefore every stage (at least during early development) is characterized by a defined cell number and a defined nuclear DNA content, independent of temperatures (for loach, e.g., see Rott & Sheveleva, 1968). The loach embryonic RNA is mainly rRNA (approx. 95%) stored during oogenesis, and its amount does not change during

early development (see Neyfakh & Timofeeva, 1977). Therefore the temperature-dependent modulations of ODC and polyamines in embryos are unlikely to be determined by changes in nucleic acid content.

It cannot be excluded, however, that the temperature shifts act on such parameters as pH or concentration of ions and other metabolites. Indirect effects of these factors on amount of ODC seem more likely than an immediate selective effect of temperature shift on translation or degradation of ODC molecules.

It seems reasonable that the role of these changes in ODC activity is to maintain different concentrations of polyamines in cells. Drastic changes in the ODC activity occur within 2–3 h after the temperature shift, but it takes 20–30 h to reach the new polyamine concentrations. We can expect that for many species of poikilothermic animals there exists an optimal concentration of polyamines for each temperature. When the temperature changes, a relatively rapid 'molecular' adaptation takes place by means of a change in ODC activity, and then in polyamine concentrations.

A hypothesis may be proposed concerning the role of polyamines in such temperature adaptation. It is known that polyamines stabilize the structure of nucleic acids, as shown by an increase in denaturation temperature (see Heby, 1981; Abraham & Pihl, 1981). We may propose that there exists an optimal degree of stability required for the normal function of nucleic acids in cells. Then, in order to maintain this degree of stability the concentration of polyamines must be greater at higher temperatures and lesser at lower temperatures. The absence of temperature-dependence in ODC activity among homoiothermic animals (birds and mammals) indirectly supports this hypothesis. Oshima (1983) has shown more directly that there is a specific polyamine requirement for protein synthesis at elevated temperatures in *Thermus thermophilus* extracts, apparently to maintain the active conformation of the ribosome-mRNA-aminoacyl-tRNA ternary complex. However, direct experiments to prove the actual role of temperature-dependent changes in ODC activity and polyamines content are necessary.

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