Direct deamination of AMP, ADP, ATP and NADH by non-specific adenylate deaminase in the foot muscle of the snail *Helix pomatia*

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Homogeneous adenylate deaminase from snail foot muscle deaminated 5'-AMP, 5'-ADP, 5'-ATP and NADH with similar velocity and affinity to all substrates. At millimolar concentration NAD^+ was also deaminated to a comparable extent, but NADP^+ , NADPH and FAD were not substrates for the snail enzyme. The amount of deaminase activity per g of fresh tissue is 5–10 times greater than in the muscle of any other species studied. The activity of the snail deaminase is regulated by pH, KCl and buffer concentrations, and P_i ; however, regulation seems to be very poor in comparison with that of muscle deaminases from other species, specific to 5'-AMP. Snail enzyme appears as the first animal deaminase so far described that has such characteristics. It offers also some opportunities as an analytical tool as a consequence of its very high affinity toward adenylates.

It was found that two dehydrogenase systems in the snail (*Helix pomatia*) foot muscle were more efficient with deamino-NADH as a coenzyme than with NADH, and that crude snail muscle extracts contain an adenylate deaminase that can deaminate NADH (Stankiewicz, 1982). Although such an enzyme has not been purified previously from any animal tissue, its presence in the rat brain and liver was postulated a decade ago (Buniatian, 1970). No detailed studies on this subject, nor on the significance of deamino-NADH in dehydrogenase systems (Pullman *et al.*, 1952) or in the utilization of NADH by mitochondria (Tischler & Fisher, 1973) were undertaken. In the present paper the purification, substrate specificity and some regulatory properties of the non-specific adenylate deaminase from snail foot muscle are described. Some physiological implications of its presence in the snail muscle are also discussed.

Methods

Determination of enzymic activity

At micromolar substrate concentrations, reaction velocity was measured by following the decrease in A_{265} which accompanies the conversion of 6-aminopurine into its hypoxanthine derivative, whereas at

millimolar substrate concentrations the reaction rate was calculated from the amount of ammonia liberated, as described previously (Stankiewicz & Spychała, 1979). Details of the composition of the incubation media are given in the Table and Figure legends. For NADH the A_{340} was also monitored. No change in A_{340} was observed within the time during which NADH deamination was monitored.

Products of deamination of AMP, ADP, ATP and NADH, with standard compounds, were chromatographed on Whatman no. 1 paper sheets in closed glass chambers at room temperature. Usually chromatography was performed for 6–8 h in solvents II and III, or for 14–18 h in solvents I and IV. For other details see the legend to Table 3.

Protein concentration was measured and polyacrylamide-gel electrophoresis was performed as described by Stankiewicz *et al.* (1979).

Reagents

Adenylates, deamino- NAD^+ , deamino-NADH, dithioerythritol, Tris (Trizma), amino acids, organic phosphates and carboxylic acids were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), NAD^+ and NADP^+ from Reanal (Budapest, Hungary), NADH and imidazole from Koch-Light (Colnbrook, Bucks., U.K.), NADPH from Fluka (Buchs, Switzerland), FAD from Calbiochem (San Diego, CA, U.S.A.). Adenosine 5'-[β,γ -imido]triphosphate was from P-L Biochemicals (Milwaukee, WI, U.S.A.). Mercaptoethanol was supplied by E. Merck

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A.G. (Darmstadt, West Germany), Sepharose 6B, DEAE-Sephacel, Sephacryl S-200 and Sephadex G-25 were from Pharmacia (Uppsala, Sweden), and Amberlites IR120 and IR50 from BDH (Poole, Dorset, U.K.). The Electrophoresis kit was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Other reagents were products of Polskie Odczynniki Chemiczne (Katowice, Poland). Solutions for enzyme purification were prepared in water triple-distilled from glass, and for other experiments water was triple-distilled from quartz and deionized on a column of Amberlite IR120/IR50 (1:1).

Experimental and results

Purification and activity of snail adenylate deaminase

Active snails were collected from the surroundings of Gdańsk in the period of May–July, stored in glass aquaria and fed with lettuce for no longer than 2 days before the experiment. Foot muscle was excised, and thoroughly cut into small pieces, then homogenized with a Waring-Blendor type homogenizer for 3×20 s with 4 vol. of 0.089 M-potassium phosphate buffer, pH 6.5, containing 0.18 M-KCl and 1 mM-mercaptoethanol. After 1 h of gentle stirring at room temperature the homogenate was centrifuged for 5 min at 12000 g. Crude extract was then dialysed for 8 h against 20 vol. of water containing 1 mM-mercaptoethanol at 35°C. The precipitate was pelleted by centrifugation for 10 min at 5000 g and discarded, and supernatant was concentrated with Aquacide (Calbiochem) and applied to the DEAE-Sephacel column (2 cm $\times$ 45 cm) equilibrated with 0.05 M-imidazole/HCl buffer, pH 6.5, containing 1 M-KCl. Enzyme was eluted from the column with the same solution. Subsequently two further chromatographies were performed, on Sepharose 6B and Sephacryl S-200 columns (2.5 cm $\times$ 70 cm), with the buffer described above for elution. Active fractions were concentrated by using polyethylene glycol 6000, followed by dialysis against 300 vol. of

0.05 M-imidazole/HCl, pH 6.5, containing 1 M-KCl, 1 mM-mercaptoethanol and 55% (v/v) glycerol. In this medium the enzyme (approx. 5 mg of protein/ml) was stored at -17°C , and no loss of the catalytic activity was observed within 2 years of storage. Before the experiment the enzyme was redialysed to 0.1 M-imidazole/HCl, pH 7.0, containing 1 mM-mercaptoethanol. The yield of the purification was about 13% and the enzyme seemed to be homogeneous, as it migrated as a single band during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (result not shown).

The specific activity of the snail enzyme was the same at micro- and milli-molar 5'-AMP concentrations (Table 1) and was much higher than in any other invertebrate studied so far (Aikawa, 1959; Bishop & Barnes, 1971; Gibbs *et al.*, 1976; MacDonell & Tillinghast, 1973). With 5'-AMP as a substrate, 39 units of the enzyme activity were found per g of fresh snail muscle, compared with activities in different vertebrate species of 2.4 to 12.9 units/g of tissue measured under the same conditions (A. J. Stankiewicz, unpublished work).

Substrate specificity

As shown in Table 2, snail deaminase used as substrates mono-, di- and tri-phosphates of adenosine as well as adenosine monosulphate and adenosine 5'-monophosphoramidate. At 70 μM , ATP was deaminated at 36%, and NADH at 66%, of the rate of AMP deamination; however, NAD⁺ was effectively deaminated only at 1 mM, and NADP⁺, NADPH, FAD and CoA were not substrates. Decrease of acidity of the 5'-substituent of ribose resulted in a decrease in the rate of deamination (adenosine 5'-monophosphomorpholidate, adenosine 5'-[β,γ -imido]triphosphate), and modification of 2'- or 3'-positions on ribose resulted in lack of deamination of these derivatives (dAMP, adenosine 2',5'- and 3',5'-biphosphates, 3',5'-cyclic AMP, 2'-AMP, 3'-AMP). A time-dependence study of the reaction with AMP, ADP, ATP and NADH as

Table 1. *Purification of snail muscle adenylate deaminase*

Extract was prepared from 130 g of muscle (30 animals). Activity was measured spectrophotometrically (at 70 μM -AMP) or from the amount of ammonia liberated (15 mM-AMP) in 0.05 M-imidazole/HCl buffer, pH 6.5, containing 0.15 M-KCl. One unit of enzyme activity was defined as the amount of enzyme which deaminated 1 μmol of AMP (A) or liberated 1 μmol of ammonia (B) per min at 25°C.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)		Purification (fold)	Yield (%)
			A	B		
Crude extract	6430	3215	0.5	0.8	—	100
Supernatant after dialysis against water	2270	2110	0.93	1.2	1.9	66
Eluate from DEAE-Sephacel column	553	1930	3.5	3.6	7.0	60
Eluate from Sepharose 6B	61	749	12.6	12.2	24.0	23
Eluate from Sephacryl S-200	6	404	67.3	64.9	135.0	12.6

Table 2. *Substrate specificity of snail muscle adenylate deaminase*

Activity was measured spectrophotometrically or from the amount of ammonia liberated in 20 mM-imidazole/HCl, pH 7.0. Mean values $\pm$ s.d. from five experiments are presented. During 3 h of incubation with excess of enzyme, no free ammonia appearance was noticed with 2'-AMP, 3'-AMP, adenosine 5'-mononucleotide, NADP⁺, NADPH, FAD and CoA (all 5 mM). ADP, ATP and NADH were not deaminated in a control experiment when incubated for 2 h with skeletal-muscle AMP deaminases from man, rat, rabbit, hen, pikeperch (*Lucioperca lucioperca*), perch (*Perca fluviatilis*), tortoise (*Testudo graeca*) and crab (*Orconectes limosus*). Abbreviation: ND, not determined.

Substrate	Relative velocity at substrate concn.:		
	70 μ M	1 mM	3 mM
5'-AMP	100	100	100
ADP	95 $\pm$ 7	91 $\pm$ 12	103 $\pm$ 1
ATP	36 $\pm$ 2	84 $\pm$ 11	79 $\pm$ 12
NADH	66 $\pm$ 6	94 $\pm$ 13	ND
NAD ⁺	6 $\pm$ 1	90 $\pm$ 13	ND
Adenosine 5'-tetraphosphate	6 $\pm$ 2	ND	ND
Adenosine 5'-monosulphate	ND	ND	71 $\pm$ 20
Adenosine 5'-monophosphoramidate	ND	ND	90 $\pm$ 28
Adenosine 5'-monophosphomorpholidate	ND	ND	38 $\pm$ 5
Adenosine 5'-[β , γ -imido]triphosphate	ND	ND	32 $\pm$ 2
Adenosine 5'-hemisulphate	ND	ND	14 $\pm$ 4
2'-Deoxy-5'-AMP (dAMP)	ND	ND	10 $\pm$ 5
Adenosine 3',5'-bisphosphate	ND	ND	8 $\pm$ 2
Adenosine 2',5'-bisphosphate	ND	ND	2 $\pm$ 1
3':5'-Cyclic AMP	0.35 $\pm$ 0.1	ND	ND
2':5'-Cyclic AMP	0.13 $\pm$ 0.05	ND	ND

Table 3. *Migration distances (mm) on paper chromatography of standard substances and products of deamination of AMP, ADP, ATP and NADH by snail muscle deaminase*

Substrates at 10 mM were incubated for 2 h at 25°C in 20 mM-imidazole/HCl, pH 7.0, with excess of the enzyme. Standards were incubated for the same time without enzyme added. Paper chromatography was performed with four solvents: I, propanol conc. NH₃ (sp.gr. 0.880)/water (6:3:1, by vol.); II, satd. (NH₄)₂SO₄/butan-1-ol/0.025 M-NH₃ (160:3:40, by vol.); III, satd. (NH₄)₂SO₄/propan-2-ol/water (79:2:19, by vol.); IV, propan-2-ol:conc. HCl/water (170:41:39, by vol.). Nucleotide spots were identified under a lamp with maximum emission at 254 nm. U.v. spectra were taken with a Unicam SP. 800 spectrophotometer after spots were eluted from the paper with 2 ml of 0.1 M-HCl. Six experiments were performed with AMP, ADP, and ATP, and three with NADH. Abbreviation: ND, not determined.

	Migration distance in the solvent:				Wavelength of maximum absorbance of the eluate (nm)
	I	II	III	IV	
AMP standard	58	105	32	82	257
IMP standard	33	180	62	64	247
AMP incubated with enzyme	31	185	55	65	247
ADP standard	42	115	45	ND	257
ADP incubated with enzyme	22	225	47	ND	247
ATP standard	32	130	35	ND	257
ITP standard	19	190	85	ND	247
ATP incubated with enzyme	18	195	95	ND	247
NADH standard	69	50	33	79	257
Deamino-NADH standard	46	130	51	63	247
NADH incubated with enzyme	48	130	51	63	247

substrates resulted in a linear relation between the amount of ammonia liberated by snail deaminase and the time of incubation (results not shown). Only small amounts of phosphate were found in the incubation media after incubation of adenylates with snail deaminase for up to 2.5 h, which indicates the

absence of 5'-nucleotidase activity from the enzyme preparation used.

Migration distances of the products of the reaction with snail deaminase were the same as for hypoxanthine standard compounds, and differed from those of standard adenine derivatives (Table 3).

Fig. 1. Effect of pH on the activity of snail deaminase 5-AMP was used as a substrate at 70 μ M (a) or 5 mM (b). Reaction rate was measured in 30mM-imidazole/HCl buffer containing 0.15 M-KCl (○) or with no salt added (●). The highest activity at given KCl and substrate concentration was taken as 100% (i.e. pH 7.25 at 70 μ M-AMP with KCl added).

Maximum absorbancies of the eluates from paper were also typical for hypoxanthine purines.

Regulatory properties

The pH optimum of AMP deamination catalysed by snail deaminase was 7.25 when measured at 70 μ M-substrate in the presence of 0.15 M-KCl, but without added KCl the reaction velocity was almost the same within the pH range 6.25–7.25 (Fig. 1a). At millimolar AMP concentrations the activity slightly increased within the pH range 5.5–8.0 if KCl was present, and a plateau was observed in the absence of KCl (Fig. 1b). Similar dependence was found when deamination of ADP, ATP and NADH was studied (results not shown). Results presented in Table 4 indicate a very high affinity of all four substrates toward snail deaminase. Moreover, the enzyme was saturated at substrate concentrations as low as 100–120 μ M, and Michaelis–Menten curves were hyperbolic (results not shown). This indicates that the 'regulatory region' of substrate concentrations is very narrow for the snail enzyme, being close to the concentrations of adenylates that one

Table 4. Michaelis constants for AMP, ADP, ATP and NADH as substrates of snail adenylate deaminase Michaelis constants were calculated from Lineweaver–Burk plots. Enzyme activity was assayed spectrophotometrically in 20mM-imidazole/HCl, pH 7.0, at 20 μ M, 30 μ M, 45 μ M, 60 μ M, 80 μ M, 100 μ M and 120 μ M substrate concentrations. For AMP K_m was also measured in 0.05 M-imidazole/HCl, pH 7.0, containing 0.15 M-KCl. Mean value $\pm$ s.d. at this condition was $53 \pm 7 \mu$ M. Data are mean values from three experiments. The rate of AMP deamination at millimolar (0.5–10.0) substrate concentration was the same as at 100 μ M-AMP.

Substrate	K_m (μ M)
AMP	31 ± 9
ADP	29 ± 7
ATP	45 ± 11
NADH	73 ± 4

could expect as physiological. It was also noticed that the K_m value for AMP was higher in the presence of 0.15 M-KCl than in its absence.

Increasing the buffer concentration, as well as the presence of KCl in the medium, resulted in an almost linear decrease of catalytic activity of snail deaminase toward adenylates. The deamination of ATP was influenced to the greatest extent. The relative reaction rates at 30mM-imidazole buffer to which 70mM-KCl was added, for AMP, ADP, ATP and NADH, were 97%, 93%, 70% and 90% respectively, in comparison with the rate without KCl. Relative reaction rates in 100mM-imidazole buffer for AMP, ADP, ATP and NADH were 80%, 70%, 60% and 90% respectively of those in 30mM-imidazole.

P_i inhibited deamination of 5'-AMP only at micromolar substrate concentrations, being without any effect at millimolar AMP. In the presence of 10mM- P_i , 51%, 60%, 78% and 66% inhibition of the deamination of AMP, ADP, ATP and NADH (all 70 μ M), respectively, was observed. When KCl was added simultaneously with P_i , the degree of inhibition was lower, being 35%, 60%, 73% and 50% for AMP, ADP, ATP and NADH respectively.

Various amino acids, organic phosphates and carboxylic acids tested had no significant effect on the activity of snail deaminase (results not shown).

Discussion

The high 'free ammonia liberating potential' of the *Helix pomatia* foot muscle demands a revision of the commonly accepted opinion that this species, like other terrestrial molluscs, is exclusively uricotelic (Lee & Campbell, 1965; Campbell *et al.*, 1972), with purines as the main waste nitrogen excreted (Jeżewska *et al.*, 1963a,b; Potts, 1967; Campbell &

Bishop, 1970). Considerable production of ammonia and its diffusion through the shell was reported only in the snail *Otala lactea* as a result of urease activity (Speeg & Campbell, 1968a,b). The results in the present paper support the conclusion that in *Helix pomatia* waste nitrogen excretion, at least in part, may depend on free ammonia production in foot muscle and its diffusion through the shell, a possibility never considered previously. This less expensive, from the energetic point of view, process can be considered as a complement to purine excretion, particularly as the activity of the adenylate deaminase is the same during aestivation as during the active period (Stankiewicz, 1982). Invertebrates tend to change from ammonotelism to ureotelism under the conditions of limited water supply or starvation (Janssens, 1964; Bishop & Campbell, 1965; Balinsky *et al.*, 1967). Since *Helix pomatia* cannot synthesize urea (Newton-Linton & Campbell, 1962; Poremska & Heller, 1962), the ability to preserve a high activity of non-specific adenylate deaminase and to excrete free ammonia during aestivation (a time of limited water supply and starvation) may be of great value for this animal.

The very high affinity of snail deaminase toward all three adenylates raises the question of possible mechanisms that could prevent the enzyme from depleting muscle adenine nucleotides *in vivo*. The differences in the inhibitory effects of phosphate and KCl on the deamination of AMP and ATP can be of some importance particularly during the aestivation period. There is no information, however, on the amounts and physiological role of adenylates in the snail, or on the presence of the purine nucleotide cycle. The whole problem remains an open question.

All adenylate deaminases from animal tissues previously studied were specific for 5'-AMP (Stankiewicz *et al.*, 1980). Direct deamination of ADP and ATP is very rare. Myofibrils from rabbit heart could deaminate ADP (Webster, 1953), as did actomyosin gel from skeletal muscle of the rabbit (Deutsch & Nilsson, 1954). Non-specific ATP deaminases were isolated from taka-diastase powder (Kalcar, 1947; Minato *et al.*, 1965a,b) and *Microsporium audini* (Chung & Aida, 1967), as well as from some marine plants (Su *et al.*, 1966) and bacteria (Yates, 1969). Snail deaminase seems to be the first animal enzyme to be described with this type of substrate specificity. From all studies mentioned above, including more recent research (Zielke & Suelter, 1971; Stankiewicz *et al.*, 1980) it is clear that 2'- and 3'-positions on ribose are of particular importance for substrates of these enzymes independent of their individual specificity, a feature that has been preserved during the course of evolution.

The physiological significance of the ability to

produce deamino-NADH in the snail muscle remains obscure, but it may affect metabolism by influencing dehydrogenase systems (Pullman *et al.*, 1952; Stankiewicz, 1982), the rate of cytoplasmic NADH oxidation by mitochondria (Tischler & Fisher, 1973), or a cycle leading to amino acid catabolism as proposed by Bunatian (1970).

Finally, the enzyme described above offers interesting opportunities as an analytical tool. It has already been used successfully for the production of some hypoxanthine mono- and di-nucleotides of experimental interest from their more common and cheaper adenine derivatives (A. J. Stankiewicz, unpublished work). Owing to the unusually high affinity of the enzyme toward adenylates, it can also be used in identifying the products of adenylate metabolism.

Concluding, one should emphasize that, in contrast with higher-animal muscle deaminases, specific to 5'-AMP, and regulated precisely in a sophisticated manner by pH, univalent cations, nucleotides, organic phosphates and some other metabolites, non-specific snail enzyme is a deaminase with a very high catalytic potential, achieving full activity at relatively low (micromolar) substrate concentrations and regulated by substrates within a very narrow range of concentrations. It operates with an efficiency close to maximum in a relatively wide pH range and is not affected by any metabolic intermediates tested. Only salinity and P_i seem to be of eventual physiological significance as its effectors. Its regulation thus seems to be very poor and simple, though potentially it could be regulated more precisely as a tetrameric protein (A. J. Stankiewicz, unpublished work).

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