REVIEW ARTICLE

5'-Nucleotidase: molecular structure and functional aspects

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INTRODUCTION

5'-Nucleotidase activity was first described in heart and skeletal muscle about 60 years ago (Reis, 1934). It catalyses the hydrolysis of phosphate esterified at carbon 5' of the ribose and deoxyribose portions of nucleotide molecules (EC 3.1.3.5). Activity of 5'-nucleotidase has been described for bacteria and plant cells and the enzyme is also widely distributed in vertebrate tissues.

Enzymes hydrolysing 5'-nucleotides from the various sources display significant differences in the range of substrates hydrolysed as well as in substrate specificity. Furthermore, there occur different cellular locations. 5'-Nucleotidase activity is found not only in soluble but also in membrane-bound, surface-located form. In addition to a broad spectrum of 5'-purine and pyrimidine mononucleotides, 5'-dinucleotides and 5'-trinucleotides, or even complex nucleotides like UDP-glucose or FAD, can also be hydrolysed by the various 5'-nucleotidases. The ability to hydrolyse 5'-mononucleotides appears to be the common denominator. This raises the question as to whether the 5'-nucleotidases at different phylogenetic levels or cellular locations belong to one principal type of enzyme or if they represent different forms of enzymes (and proteins) with partially overlapping ranges of substrates. Recent analyses of the primary structures derived from cDNAs of bacteria and vertebrates have provided a means to group at least part of the enzymes so far described into one family of phylogenetically related proteins.

Whereas cytosolic 5'-nucleotidase activity controls intracellular levels of nucleoside 5'-monophosphates, surface-located 5'-nucleotidase is a major contributor to the cascade that completely hydrolyses extracellular ATP to adenosine, and thus of major pharmacological interest. The physiological function of the enzyme probably differs in various organisms and tissues, and possibly extends beyond its catalytic activity. Thus for example surface-located 5'-nucleotidase anchored to the plasma membrane by glycosyl phosphatidylinositol has been implicated in cell-matrix or cell-cell interactions, and even in transmembrane signalling. 5'-Nucleotidase can be transiently expressed in certain cell types during development and the activity of 5'-nucleotidase in cultured cells can be regulated by external factors. It may even be released into the extracellular medium.

Various aspects of 5'-nucleotidase molecular properties and function have been reviewed (Bodansky & Schwartz, 1968; Drummond & Yamamoto, 1971; Arch & Newsholme, 1978; Fox, 1978; Riordan & Forstner, 1978; Stone, 1981; Pearson, 1985, 1987; Dieckhoff *et al.*, 1986a; Dornand *et al.*, 1986; Gutensohn & Rieger, 1986; Karnovsky, 1986; Kreutzberg *et al.*, 1986; Luzio *et al.*, 1986, 1987; Newby & Worku, 1986; Slakey *et al.*, 1986; Widnell *et al.*, 1986; Grondal & Zimmermann, 1988). Alterations of 5'-nucleotidase levels in a considerable number of diseases have been recognized (Sunderman, 1990). The present review focuses on recent developments in the analysis of the molecular structure and of functional aspects of 5'-mononucleotide-hydrolysing enzymes. It suggests a classification

and divides the vertebrate 5'-nucleotidases into four distinct groups according to biochemical properties and cellular location.

STRUCTURE OF 5'-NUCLEOTIDE-HYDROLYSING ENZYMES AS DEDUCED FROM cDNA

Vertebrate enzymes

The primary structure of 5'-nucleotidase has now been determined for two mammalian species (rat liver, Misumi *et al.*, 1990a; human placenta, Misumi *et al.*, 1990b) and an Elasmobranch fish (brain of the electric ray, *Discopyge ommata*, Volkandt *et al.*, 1991) (Fig. 1; Table 1). In all three cases the mature protein is expected to consist of 548 amino acids with a molecular mass of about 61 kDa. Variations in the length of the signal peptide yield small differences in the lengths of the presumptive open reading frames (576, rat liver; 574, human placenta; 577, electric ray brain). The C-termini contain a stretch of uncharged and hydrophobic amino acid residues which are presumably exchanged for a glycosyl phosphatidylinositol (GPI) anchor bound to Ser-523 (Ogata *et al.*, 1990). The human placenta and electric ray brain enzymes contain four potential N-linked glycosylation sites; the rat liver enzyme contains five.

The two mammalian enzymes display nearly 90% amino acid identity, whereas the electric ray 5'-nucleotidase displays a 61% identity with either mammalian enzyme. The differences in the amino acid sequence between the mammalian and the ray enzymes are rather randomly distributed. Some stretches of amino acid sequence in the ray enzyme are identical to the rat but different from the human enzyme while others are identical to the human but different from the rat enzyme (Fig. 1) (Volkandt *et al.*, 1991). The conservative character of the protein structure is obvious when hydropathy profiles are compared (Fig. 2). It not only demonstrates the expected correspondence of the hydrophobic domains at the N-terminal (signal peptide) and C-terminal (site for cotranslational cleavage and attachment of GPI anchor) sequences. It also shows the high similarity in the overall pattern of hydrophobic and hydrophilic domains. It should be noted that the identity of hydropathy profiles does not always correlate to amino acid identity. Five to seven domains of the protein reveal a particularly high conservation in amino acid sequence during evolution (Fig. 3). The rat/human identities in these domains generally amount to 90–100% (not shown) while ray/rat identities range from 71% to 100%.

Fourier transform infrared spectroscopy further revealed insights into the secondary structure of 5'-nucleotidase from bull seminal plasma (Fini *et al.*, 1992). A quantitative analysis of the amide I components revealed that the protein contains mostly β -sheet structure (54%) with 18% α -helix, 22% β -turns and 5% unordered structure. According to our computer aided calculations the predicted secondary structure of the electric ray 5'-nucleotidase includes 49.2% β -sheets, 32.7% α -helix, 9.5% β -turns and 8.4% unordered structure. This is rather similar to the

	-29	+1	29
ray	MPRVPSASATGSSALLSLLCAFSLGRAAP-	FQLTILHTNDVHARVEETNQDSGKCF	TQS-
rat	-.-.A.ATAPKWL..A.SALLP.WPT.KS	WE.....S.L.Q.SD..T..LNA.L	
hum	.C--.R.ARAPATL..A.GAVL--WP..GA	WE.....S.L.Q.SE..S..VNA.R	
	30		88
ray	-FAGVARRWTKIEELRARDKNVLLLDAGDQYQGTIWFNYKGAEEAHFIEAVGYNAMALG		
rat	CVG....LF..VQQI.KEEP.....TV...L.V...MNL..D.....		
hum	CMG....LF..VQQI.RAEP.....TV.....V...MN.LR.D.....		
	89		148
ray	NHEFDNGAEGLLDPFLLNVSPVLSANLEQGEDQVPSLIGYYKPSTVLDVNGEKIGVVGY		
rat	V...I..L.R..K..I.....IKARGPLA.QIS.L.L.YK..S.G..VV.I...		
hum	V...IE.L.KEAK..I.....IKAKGPLASQIS.L.L.YK..P.GD.VV.I...		
	149		208
ray	TSKETPTLSSPGPHLIFKDEIQAVQHEVDILVSQGIDKIIALGHSGFETDKLIAQKVRGV		
rat	F..N..TN.V.E..VT.L.P...K.KTLNVN.....M.....		
hum	F..N..TN.V.E..T.L.P...K.KTLNVN.....M.....		
	209		267
ray	DVVVGHSNTFLYTGKAPSNDVPVGPYPFLVNSDDQRTIPVVQAYAYGKYLGYLKLTFD-		
rat	T.....NP..KE..A.K...I.T...G.KV.....F.....VE..D		
hum	T.....NP..KE..A.K...I.T...G.KV.....F.....IE..E		
	268		327
ray	KGEVIKREGNPILLNSSIIQDPVLLAEVNKWKESLANFGKEVIGRTVVYLNGTTEECRNR		
rat	..N.VTSY.....T.RE.AAIK.DI.Q.RIK.D.YSTQEL...I.....SAQ...F.		
hum	R.N..SSH.....PE..SIK.DI..RIK.D.YSTQEL.K.I...D.SSQS..F.		
	328		387
ray	ECNMGNLICDAMIQQNIRNPDEKFWNHVSICIFQGGGIRAPINEQNGTIQVDSLAVLP		
rat	NN.L.H...M.....M..VN.....S..D.R.....TWEN.A....		
hum	NN.L.HT..M.....M..LN.....S..D.R.....TWEN.A....		
	388		447
ray	FGSTIDLLEVYGSTLRAAFDHSVRRYGQNTGEFLQVSGIQVQFNLKRPPGSRVVKIDVLC		
rat	..G.F..VQLK....KK..E...H....S.....G..H.VYDIS.K.WD...QLK...		
hum	..G.F..VQLK....KK..E...H....S.....G..H.VYD.S.K..D...L....		
	448		507
ray	ADCRVPHYQPLLDNKIYKIVTNSYIAEGGDGFTMLKNERLRYDTGSTDISVVSSYIKQMK		
rat	TK....I.E...EMD.V..V.LP...VN.....Q.I.D.L.KH.S.DQ.....E..SK..		
hum	TK....S.D..KMDEV..VILPNFL.N.....Q.I.D.L..H.S.DQ..N...T..SK..		
	508	548	
ray	VVYPAVEGRILFVENSATLPIINLKIGLSLFAFLTWFLHCS		
rat	.I.....K.---S.ASHYQGSFPLII.SFWAVILVLYQ		
hum	.I.....K.---STGSHCHGSFSLIF.SLWAVI.VLYQ		

Fig. 1. Manual alignment of the deduced amino acid sequences from cDNA encoding (ecto-) 5'-nucleotidase from electric ray brain (electric lobe, *Discopyge ommata*), rat liver and human placenta

The amino acid residues are numbered on top of the respective amino acid. The signal peptides have small capital letters and were assigned negative numbers. Amino acids identical to the ray enzyme are indicated by dots, amino acids common to all three enzymes are tinted, and introduced gaps are indicated by hyphens. The serine residues at position 523 terminating the amino acids of the mature membrane-bound protein are marked by an asterisk. Small stretches of amino acids that are conserved between animal and bacterial 5'-nucleotidases (Table 1, Fig. 3) are in boldface type. Potential N-glycosylation sites are coloured red. Sequences are from Misumi *et al.*, (1990a) (rat liver), Misumi *et al.* (1990b) (human placenta) and Volkandt *et al.* (1991) (electric ray brain). (Modified from Volkandt *et al.*, 1991).

Table 1. Identity of ray 5'-nucleotidase with other enzymes (without signal peptide)

References: 1, Misumi *et al.* (1990b); 2, Misumi *et al.* (1990a); 3, Burns & Beacham (1986a); 4, Tamao *et al.* (1991); 5, Burns & Beacham (1986b); 6, Liu *et al.* (1986).

Protein	Source	Identity (%)	Number of amino acids	Reference
5'-Nucleotidase	Rat liver	61.5	548	[1]
5'-Nucleotidase	Human placenta	61.1	548	[2]
5'-Nucleotidase, UDP-sugar hydrolase (<i>ushA</i>)	<i>E. coli</i>	22.7	525	[3]
5'-Nucleotidase	<i>V. parahaemolyticus</i>	22.6	539	[4]
<i>ushA</i> ⁰ (silent)	<i>S. typhimurium</i>	22.0	527	[5]
Phosphodiesterase	<i>E. coli</i>	17.9	646	[6]

calculated structure of the electric ray GPI-linked form of acetylcholinesterase.

It is apparent from both immunochemical and biochemical evidence that the cDNAs isolated so far correspond to the membrane-bound and surface-located form of the enzyme. Since only one sequence has been obtained for a given species it remains to be elucidated whether there exist tissue-specific distinct gene products as in the case of, for example, alkaline phosphatase (E.C. 3.1.3.1) (Kenny & Turner, 1987). It can be expected that the cytoplasmic forms of 5'-nucleotidase are encoded by additional genes.

Bacterial enzymes

Enzymes capable of hydrolysing 5'-mononucleotides are also expressed in bacteria. These reveal substrate specificities which are rather different from those of vertebrate enzymes and whose general functional significance might differ. Isolation of the genes revealed sequence identities between the bacterial enzymes. A Cl⁻-dependent 5'-nucleotidase from *Vibrio parahaemolyticus* (*nutA*, Tamao *et al.*, 1991) shares 60% identity with the UDP-sugar hydrolase of *Escherichia coli* (Burns & Beacham, 1986b), an enzyme also capable of hydrolysing a variety of 5'-nucleotides including 5'-mononucleotides. A silent gene potentially coding for UDP-sugar hydrolase in *Salmonella typhimurium* (*ushA*⁰) (Burns & Beacham, 1986a) is 87% identical with that of *Escherichia coli* (*ushA*). All these enzymes carry signal peptide sequences and the number of amino acids in the open reading frame varies between 550 and 560.

Interestingly, a comparison of the deduced primary structures of vertebrate and bacterial enzymes disclosed sequence identities which indicate common ancestry (Fig. 3). Overall amino acid sequence identities are only slightly more than 20%. However, an analysis of those amino acid clusters that reveal high identity in ray and mammalian enzymes shows that also in bacterial and vertebrate 5'-nucleotidases the same regions tend to be highly conserved. In addition, sequence identities are observed in vertebrate 5'-nucleotidase and an enzyme from *Escherichia coli* (*cpdB*; Liu *et al.*, 1986) capable of hydrolysing 3'-nucleotides as well as 2',3'-cyclic phosphodiesterases (Anraku, 1964a,b). It can be expected that the conserved sequence clusters are of considerable functional significance, possibly including one or more substrate-binding sites. The notion of homology in enzyme structures is further supported by the remarkable similarity of the hydropathy profiles of the vertebrate and prokaryotic proteins (Fig. 2). Whereas the hydropathy profile for the (periplasmic) 5'-nucleo-

Fig. 2. Hydropathy plots of ecto-5'-nucleotidase from electric ray brain, rat liver and *Escherichia coli* (*ushA* gene; Burns & Beacham, 1986b) (scanning window 11 amino acids).

The cleavage sites of the signal peptide (arrow) and the C-terminal domain (arrow head) are marked. The domains represented in Fig. 3 are marked by asterisks. Note the absence of a hydrophobic C-terminal sequence from the bacterial enzyme. (Modified from Volkandt *et al.*, 1991).

tidase (*ushA*) of *Escherichia coli* reveals an N-terminal pattern similar to the vertebrate enzyme, the C-terminal region lacks the hydrophobic region which in vertebrates is exchanged for the GPI-anchor.

BIOCHEMICAL PROPERTIES: ANIMALS

An attempt is made to group animal 5'-nucleotidases according to their cellular location and molecular and kinetic properties into four forms (Table 2): one membrane-bound form (ecto-5'-nucleotidase, e-N) and three soluble forms. One of the soluble forms (e-N₁) appears to be derived from the GPI-anchored ecto-5'-nucleotidase and has presumably an extracellular location. The two cytosolic soluble forms have similar characteristics but can be clearly differentiated on the basis of their preferential affinities for AMP (cytoplasmic 5'-nucleotidase-I, c-N-I) and IMP (cytoplasmic 5'-nucleotidase-II, c-N-II). Nearly all studies are derived from vertebrates and within this group the general characteristics of each form of enzyme remain rather constant.

Membrane-bound form (e-N)

General properties. One form of mammalian 5'-nucleotidase is membrane-bound. It occurs as a surface-located ectoenzyme and is anchored to the plasma membrane via GPI at its C-terminus (see Fig. 5). Membrane-bound 5'-nucleotidase has been partially or fully isolated from a wide variety of vertebrate cellular sources (Table 2). It hydrolyses exclusively nucleoside 5'-monophosphates, showing no activity for 2'- and 3'-monophosphates. Reports vary concerning the capability of ecto-5'-nucleotidase to hydrolyse 2-deoxyribose compounds. If so, these were hydrolysed less effectively. Hydrolysis of 5'-AMP is stereoselective. The L-enantiomer is not hydrolysed (Cusack *et al.*, 1983). Based on the $V_{\max}/K_m$ ratio, 5'-AMP is the best substrate. K_m values for AMP are in the low micromolar range.

Fig. 3. Identity in amino acid sequence within certain domains of 5'-nucleotidase (5'-NUC) between animal (ecto-5'-nucleotidase) and various bacterial sources

Conserved domains are boxed (red) and the amino acids are numbered at the edges. Numbers in boxes represent % identity within the sequence indicated. Numbers in parentheses represent overall sequence identity. Bacterial enzymes are from *Vibrio parahaemolyticus* and *Escherichia coli*; Cl⁻-5'-NUC, chloride-stimulated 5'-nucleotidase; *ushA*, UDP-sugar hydrolase (5'-nucleotidase); 2',3'-cPDE, 2',3'-cyclic phosphodiesterase; C, C-terminus of uncleaved protein; N, N-terminus of mature protein; S, serine residue at position 523; SP, signal peptide. (Modified from Volkandt *et al.*, 1991).

ADP, ATP and adenosine 5'-[α,β -methylene]diphosphate are highly effective competitive inhibitors whereas inhibition by concanavalin A is non-competitive. Inhibition constants generally are in the low micromolar range; those for adenosine 5'-[α,β -methylene]diphosphate are even in the nanomolar range. Inorganic phosphate does not effectively inhibit the enzyme and maximal enzyme activity is obtained at the slightly alkaline pH range of 7–8. Interestingly, methylxanthines like theophylline and caffeine are also inhibitors of 5'-nucleotidase (Fredholm *et al.*, 1978; Heyliger *et al.*, 1981). Enzyme activity does not depend on added divalent cations but it can be increased by the addition of millimolar concentrations of Mg²⁺. Metal ions such as Pb²⁺ or Hg²⁺ inhibit enzyme activity and have been related to a decrease in 5'-nucleotidase activity on chronic intoxication (Ong *et al.*, 1990). The natural metal ligand of ecto-5'-nucleotidase is presumably Zn²⁺ since the purified enzyme for chicken gizzard contains tightly associated zinc at a molar ratio zinc/protein of 2 (Fini *et al.*, 1990). Correspondingly, 5'-nucleotidase activity is decreased in lymphocytes of human subjects with zinc deficiency (Metfah *et al.*, 1991). In the electric ray the cysteine residues at positions 324, 329 and 358, and a histidine residue at position 354, may function as potential Zn²⁺-binding sites (Volkandt *et al.*, 1991).

There is evidence that the substrate specificity of animal ecto-5'-nucleotidase is broader than originally anticipated. For a nearly homogeneous preparation of 5'-nucleotidase from human placental trophoblastic microvilli, FAD pyrophosphatase ac-

tivity has been reported (Lee & Ford, 1988). 5'-Nucleotidase from a placental microsome fraction (Fox & Marchant, 1976) as well as purified 5'-nucleotidase from the electric ray electric organ (Volkandt *et al.*, 1991) contain activity of UDP-glucose hydrolase. This is further support for a homology between bacterial 5'-nucleotidase (*ushA* gene) and vertebrate ecto-5'-nucleotidase (Volkandt *et al.*, 1991).

Ecto-5'-nucleotidase is an $\alpha\alpha$ -dimer with interchain disulphide bridges. This is demonstrated by analysis of the apparent M_r under non-reducing and reducing conditions (Dieckhoff *et al.*, 1985; Grondal & Zimmermann, 1987), by chemical cross-linking (Naito & Lowenstein, 1981; Harb *et al.*, 1983; Bailyes *et al.*, 1984; Buschette-Brambrink & Gutensohn, 1989), and by dissociation of the dimeric protein with dithiothreitol (Fini *et al.*, 1985). Intact disulphide bridges are essential for enzyme activity, and thiol reagents inactivate the enzyme (Worku *et al.*, 1984; Fini *et al.*, 1985). But it is not clear how many thiol groups are involved in dimer formation (Fini *et al.*, 1988). The apparent molecular masses of the monomer vary between 60 and 80 kDa depending on source and gel system used. The occasional observation of an association of lower-molecular-mass components with the α -subunit (Bailyes *et al.*, 1982, 1984; Buschette-Brambrink & Gutensohn, 1989) needs further analysis. In the human the enzyme is encoded on chromosome 6 (Boyle *et al.*, 1988).

On the basis of the inactivation of 5'-nucleotidase by group-specific reagents it has been suggested that 5'-nucleotidase

Table 2. Properties of the four types of 5'-nucleotidase in vertebrate tissue

The classification of nucleotidases is explained in the text. Abbreviations used: Ap[CH₂]p, adenosine 5'-[α,β -methylene]diphosphate; 2,3-BPG, 2,3-bisphosphoglycerate; ConA, concanavalin A; -, not determined. References are as follows: 1, Ipata (1968); 2, Widnell & Unkeless (1968); 3, Huang & Keenan (1972); 4, Evans & Gurd (1973); 5, Dornand *et al.* (1978); 6, Doss *et al.* (1979); 7, Fukui & Sichi (1981); 8, Naito & Lowenstein (1981, 1985); 9, Baillyes *et al.* (1982, 1984); 10, Harb *et al.* (1983); 11, Camici *et al.* (1985); 12, Dieckhoff *et al.* (1985); 13, Grondal & Zimmermann (1987); 14, Thompson *et al.* (1987); 15, Wada *et al.* (1987); 16, Stefanovic *et al.* (1988); 17, Buschette-Brambrink & Gutensohn (1989); 18, Le Hir *et al.* (1989a); 19, Stochaj *et al.* (1989b); 20, Fini *et al.* (1990); 21, Misumi *et al.* (1990a); 22, Flocke & Mannherz *et al.* (1991); 23, Volkmandt *et al.* (1991); 24, Mallol & Bozal (1983); 25, Fini *et al.* (1983, 1985, 1991); 26, Fritzon *et al.* (1986); 27, Madrid-Marina & Fox (1986); 28, Thompson *et al.* (1987); 29, Wada *et al.* (1987); 30, Le Hir & Dubach (1988); 31, Lisanti *et al.* (1989); 32, Spychala *et al.* (1988); 33, Stochaj *et al.* (1989b); 34, Lai & Wong (1991a); 35, Picc & Le Hir (1991); 36, Wortmann *et al.* (1991); 37, Itoh & Oka (1985); 38, Newby (1988); 39, Itoh *et al.* (1986); 40, Truong *et al.* (1988); 41, Skladanowsky & Newby (1990); 42, Yamazaki *et al.* (1991); 43, Itoh & Tsushima (1971); 44, Naito & Tsushima (1976); 45, van den Berghe *et al.* (1977); 46, Itoh *et al.* (1978, 1986); 47, Itoh (1981); 48, Carson & Wasson (1982); 49, Worku & Newby (1982); 50, Keller *et al.* (1985); 51, Iizasa *et al.* (1986); 52, Pinto *et al.* (1986); 53, Tsushima (1986); 54, Oka *et al.* (1988); 55, Spychala *et al.* (1988, 1989); 56, Bontemps *et al.* (1988, 1989); 57, Johnson & Fridland (1989); 58, Skladanowski *et al.* (1989); 59, Madrid-Marina (1990); 60, Itoh & Yamada (1990, 1991).

Type of nucleotidase	Apparent molecular mass (kDa)		Apparent K_m for:		Inhibition by (i), activation by (a), or no effect of (o)				Hydrolysis of p-nitro phenyl phosphate		Metal content	pH optimum	Membrane anchor	Substrates hydrolysed	Substrate preferred	References
	Native	Subunit	AMP (μ M)	IMP (μ M)	ATP	ADP	P _i	AP ₄ A	Ap[CH ₂]p	2,3-BPG	ConA					
e-N, e-N _s (ecto-form)	120-160	60-80	1-50 (μ M)	10-50 (μ M)	i	i	o	-	i	-	i	7-8	GPI	Nucleoside 5'-mono-phosphates, UDP-glucose (<i>Torpedo</i>)	AMP	e-N [1-23]; e-N _s [24-36]
c-N-I (AMP-cytosolic)	150	40	2-8 (mM)	-	o	a*	i	-	o	-	o	6.5	-	Nucleoside 5'-monophosphates and their 2'-deoxyribo derivatives	AMP	[37-42]
c-N-II (IMP-cytosolic)	200-265	52-70	1-15 (mM)	0.1-0.6 (mM)	a*	a*	i	a	o	a	o	6-7	-	Nucleoside 5'-monophosphates and their 2'-deoxyribo derivatives	IMP	[43-60]

* The activator shifts K_m values into the micromolar range.

Fig. 4. Qualitative scheme representing the general kinetic characteristics of ecto-5'-nucleotidase (e-N) (or low- K_m soluble 5'-nucleotidase, e-N_s), the AMP-preferring cytoplasmic enzyme (c-N-I), and the IMP-preferring cytoplasmic enzyme (c-N-II)

Note the varying scales on the abscissa. The Figure demonstrates the principle differences in affinity towards AMP or IMP and in the effects of ADP, ATP or P_i . The low- K_m ecto-5'-nucleotidase (e-N) is inhibited by ATP. The high- K_m AMP-preferring enzyme (c-N-I) is activated by ADP only, whereas the IMP-preferring enzyme (c-N-II) is activated by both ADP or ATP. Both enzymes are inhibited by P_i . As competitive inhibitor ATP increases the K_m value for e-N. However, ADP and/or ATP greatly decrease the K_m values and increase V_{max} for the cytoplasmic enzymes c-N-I and c-N-II. This behaviour seems to be of principal importance regarding enzyme function in intact tissue. (Deduced from the literature quoted in the text. Key references are in Table 2.)

belongs to a group of histidine phosphatases that includes glucose-6-phosphatase and acid phosphatase (Worku *et al.*, 1984). It would thus differ from the serine hydrolases alkaline phosphatase and acetylcholinesterase (EC 3.1.1.7). It should be noted, however, that a number of hydrolases (including acetylcholinesterase) possess a histidine in their catalytic triad (Sussman *et al.*, 1991).

GPI anchor. Three lines of evidence support the notion that vertebrate membrane 5'-nucleotidase is anchored to lipid via GPI.

(1) In a variety of tissues and cellular systems it can, at least partly, be released from the membranes by treatment with phosphatidylinositol-specific phospholipase C (EC 3.1.4.3) (Low & Finean, 1978; Taguchi & Ikezawa, 1978; Shukla *et al.*, 1980; Panagia *et al.*, 1981; Grondal & Zimmermann, 1987; Thompson *et al.*, 1987, 1989, 1990; Hooper & Turner, 1988; Klip *et al.*, 1988; Lisanti *et al.*, 1989; Stochaj *et al.*, 1989a,b; Tanaka *et al.*, 1989; Klemens *et al.*, 1990; Torres *et al.*, 1990; Misumi *et al.*, 1990a). It also has been suggested that 5'-nucleotidase may be released from skeletal muscle membranes by insulin-activated endogenous phospholipase C (Klip *et al.*, 1988).

(2) The detergent-solubilized and isolated enzyme contains

equimolar amounts of *myo*-inositol (Baillyes *et al.*, 1990; Klemens *et al.*, 1990).

(3) Using sequence information from an isolated C-terminal fragment of rat liver and human placenta 5'-nucleotidase it could be demonstrated (Ogata *et al.*, 1990; Misumi *et al.*, 1990b) that mature 5'-nucleotidase ends at Ser-523. It has lost the predicted C-terminal extension from 524 to 548 which has been cleaved and replaced by GPI.

Thus, 5'-nucleotidase belongs to a large group of surface-located proteins which can be anchored by GPI, such as alkaline phosphatase, acetylcholinesterase, aminopeptidase P, Thy-1 or the variant surface glycoprotein of *Trypanosoma* (Low, 1990; Turner *et al.*, 1991). Like other GPI-anchored proteins, 5'-nucleotidase can be solubilized effectively solely with detergents of high critical micelle concentrations (octylglycoside, CHAPS or sodium deoxycholate) (Hooper & Turner, 1988). The phospholipid anchor rather than a portion of the polypeptide chain seems to be responsible for the 5'-nucleotidase/detergent interaction.

The primary molecular structure of the GPI anchor has now been determined for a number of protozoan and vertebrate proteins. These include the variant surface glycoprotein of *Trypanosoma*, acetylcholinesterase, and Thy-1 (Low, 1989;

Fig. 5. Molecular model of ecto-5'-nucleotidase (e-N) and the soluble forms (e-N_s) derived from it

The GPI-anchored form exists as a dimer with the two subunits linked by at least one disulphide bridge. Varying with species each subunit may carry four or five N-linked oligosaccharide chains and in some species bind the monoclonal HNK-1 antibody. Soluble forms can exist as dimers or tetramers. By means of specific antibodies their origin from the GPI-anchored form can be demonstrated. The glycan (red) remains attached to the protein. Abbreviations: EtN, ethanolamine.

Ferguson & Homans, 1990). Generally, anchored proteins are connected to GPI through the α -carboxyl of the C-terminal amino acid via amine-linked phosphoethanolamine. In turn the phosphoethanolamine is linked to a glycan consisting of mannose and glucosamine, and the glucosamine is glycosidically linked to an inositol-containing phospholipid. The GPI anchors of both rat liver and human placental 5'-nucleotidase contain (molar ratios in parentheses): ethanolamine (2), mannose (3), glucosamine (1), and inositol (1). Fatty acids include stearic acid, myristic acid and palmitic acid (2 mol in total) (Ogata *et al.*, 1990; Misumi *et al.*, 1990b). It is remarkable that they lack the galactose and N-acetylglucosamine moieties identified in a number of other anchors (Ogata *et al.*, 1990). Of the two ethanol-

amines one is presumably involved in the linkage of GPI to the α -carboxyl group of Ser-523. The other one could be phosphodiester-linked to a mannose residue of the glycan core.

The physiological implication of membrane anchorage by GPI is not yet fully understood. It could facilitate the release of protein from the GPI-anchor by endogenous phospholipases (Fox *et al.*, 1987; Davitz *et al.*, 1989), tight membrane packing of proteins or also production of free membrane lipids (Saltiel, 1990). An attractive possibility has recently been developed using cultures of polarized cells (epithelial cells). The GPI-moiety may play a role as a signal for targeting the attached protein selectively to the apical membrane surface (Lisanti & Rodriguez-Boulan, 1990).

Table 3. Sources and properties of 5'-nucleotidase in bacteria

References are as follows: 1, Bengis-Garber & Kushner (1981); 2, Bengis-Garber (1985); 3, Sakai *et al.* (1987); 4, Itami *et al.* (1989); 5, Tamao *et al.* (1991); 6, Melo & Glaser (1966); 7, Glaser *et al.* (1967); 8, Neu (1967a); 9, Neu (1968); 10, Mauck & Glaser (1970); 11, Yagil & Beacham (1975); 12, Shiio & Ozaki (1978); 13, Beacham & Wilson (1982); 14, Shiio & Ozaki (1978); 15, Ozaki & Shiio (1979).

Source	Cellular location	Substrate	Requirement for metal ion	References
<i>Photobacterium</i> , <i>Vibrio</i>	Membrane-bound	Nucleoside 5'-tri-, 5'-di- and 5'-mono-phosphates	(+)	[1-5]
<i>Escherichia</i> spp., <i>Bacillus subtilis</i> , <i>Salmonella westlaco</i>	Periplasmic	Nucleoside 5'-tri-, 5'-di- and 5'-mono-phosphates (also deoxyribonucleotides), nucleoside diphosphate sugars	(+)	[6-13]
<i>Bacillus subtilis</i>	Soluble	Nucleoside 5'-mono-phosphates	(+)	[14,15]

Other modes of integration into the lipid bilayer? 5'-Nucleotidase is less effectively released from membranes by GPI-specific phospholipase C or D than are other ecto-enzymes such as alkaline phosphatase or acetylcholinesterase (Low & Finean, 1978; Shukla *et al.*, 1980; Hooper & Turner, 1988; Grondal & Zimmerman, 1987; Stochaj *et al.*, 1989b). Generally, more than 50 % of the enzyme remains in membrane-bound form. This could indicate variations in the GPI anchor, possibly involving acylation of the inositol (Rosenberry *et al.*, 1990). In addition, membrane anchoring of 5'-nucleotidase via transmembrane segments has been suggested (Zachowski *et al.*, 1981; Dieckhoff *et al.*, 1984; Klemens *et al.*, 1990). In this respect 5'-nucleotidase perhaps resembles other surface proteins which possess splicing variants with different kinds of membrane anchorage, such as acetylcholinesterase (Chatonnet & Lockridge, 1989) or N-CAM (He *et al.*, 1987). At present, and as long as the structure of the gene has not been elucidated, the possibility still needs to be considered that part of 5'-nucleotidase has a membrane-integral anchor. However, after detergent solubilization, purification and reconstitution into phospholipid vesicles, membrane-bound 5'-nucleotidase (from chicken gizzard or human pancreatic carcinoma cells) is completely transformed into a hydrophilic form by GPI-specific phospholipase C or D (Stochaj *et al.*, 1989b). Either the structure of the anchor is modified on enzyme isolation or steric hindrance attenuates the attack of GPI-specific phospholipase and thus the release of GPI-bound 5'-nucleotidase in intact membranes.

Soluble forms

To date, soluble forms have not been characterized as extensively as the ecto-enzyme. A number of studies have been performed on crude tissue extracts. The varying contributions of the different soluble 5'-nucleotidases as well as other not-yet-specified nucleotidases possibly have influenced the results obtained.

High- K_m AMP- and IMP-preferring forms (c-N-I, c-N-II). These forms are thought to be involved in the control of intracellular levels mainly of IMP and AMP. They are specific for nucleoside 5'-mononucleotides and may also hydrolyse deoxyribonucleotides. Nucleoside-2'- or 3'-phosphates and ribose 5'-phosphates are not hydrolysed.

One form (c-N-II) has a strong preference for IMP over AMP. Sometimes it is referred to as IMPase. Whereas hyperbolic kinetics are observed for IMP those for AMP tend to be sigmoidal. K_m values for IMP range from 0.1 to 0.6 mM. Those

for AMP are even higher and in the range 1–15 mM. However, millimolar concentrations of ATP and ADP considerably increase both maximal velocity and affinity for AMP. The enzyme is inhibited by P_i but unaffected by adenosine 5'-[α,β -methylene]diphosphate. It shows apparent inhibition by inosine and 2',3'-dideoxyinosine, which results from the exchange of the phosphate moiety between the nucleoside and IMP (Worku & Newby, 1982; Keller *et al.*, 1985; Johnson & Fridland, 1989; Skladanowski *et al.*, 1989; Tozzi *et al.*, 1991). Glycerate 2,3-bisphosphate (Bontemps *et al.*, 1988, 1989; Itoh & Yamada, 1990; Tozzi *et al.*, 1991) and also the diadenosine polyphosphates Ap_3A , Ap_4A and Ap_5A are potent stimulators (Pinto *et al.*, 1986; Itoh & Yamada, 1990). The slightly acidic pH optimum of about 6.5 is a further criterion to differentiate this soluble form clearly from the membrane-bound form. The enzyme has been identified in a number of vertebrate tissues including heart (Table 2) and also in the invertebrate *Artemia* (Pinto *et al.*, 1986, 1987). Estimates of the molecular mass derived from chromatography experiments range between 200 and 265 kDa. Values obtained for the subunit molecular mass are 42 kDa (*Artemia*), and 52–70 kDa for vertebrate tissues. The enzyme appears to be a homo-oligomer, presumably a tetramer.

A second soluble form of the enzyme (c-N-I) reveals a preference for AMP over IMP. Its properties are rather similar to those of the IMP-preferring enzyme except that the purified form is stimulated only by ADP and not by ATP. Another important difference between the two enzymes is that the K_m value for AMP of c-N-I is shifted into the low micromolar range by micromolar concentrations of ADP (Yamazaki *et al.*, 1991). It is expected that the two enzymes carry binding sites for ATP and/or ADP in addition to AMP. Whereas the IMP-preferring form has a broad tissue distribution, the AMP-preferring enzyme has to date been characterized only in vertebrate heart (Table 2). Here the two enzymes are expressed simultaneously. It is noteworthy that in pigeon heart the activity of ecto-5'-nucleotidase is very low (Meghji *et al.*, 1988; Skladanowski & Newby, 1990). The two enzymes may be separated using phosphocellulose columns and have been termed 5'-nucleotidase I (N-I, preferring AMP) and 5'-nucleotidase II (N-II, preferring IMP) (Truong *et al.*, 1988; Skladanowski & Newby, 1990). In this review they are referred to as cytoplasmic forms c-N-I and c-N-II in order to differentiate them from the soluble form derived from the GPI-anchored enzyme (e-N_s). The subunit molecular mass (40 kDa) of c-N-I isolated from rabbit heart (Yamazaki *et al.*, 1991) is significantly different from that of ecto-5'-nucleotidases and

preliminary sequence data reveal no homology with the plasma membrane enzyme. Since the molecular mass of the enzyme from pigeon heart determined by gel-exclusion chromatography is about 150 kDa (Skladanowski & Newby, 1990), the enzyme may exist as a homo-oligomer, possibly a tetramer.

Low- K_m soluble form (e- N_s). Yet another form of soluble 5'-nucleotidase has recently received increasing attention. This enzyme has a preference for AMP. Also its other characteristics (inhibition by ATP, ADP, adenosine 5'-[α,β -methylene]-diphosphate, concanavalin A) are basically identical with those of the membrane-bound ecto-form. Such preparations have been described for a variety of vertebrate tissues and cellular systems (Table 2). Its *N*-terminal amino acid sequence was found to correspond closely to that of the ectoenzyme (Klemens *et al.*, 1990). Like ecto-5'-nucleotidase this form can be inhibited by xanthine derivatives (Fredholm & Lindgren, 1983). Furthermore, it shares the general kinetic characteristics of ecto-5'-nucleotidase, and also the tight association of zinc (Fini *et al.*, 1990). It is recognized and even inhibited by antibodies obtained against ecto-5'-nucleotidase (Zekri *et al.*, 1988; Stochaj *et al.*, 1989b; Piec & Le Hir, 1991). Apparent molecular masses are similar or identical to those of the membrane-bound form. 5'-Nucleotidase from the venoms of snakes like *Bothrops*, *Crotalus*, *Haemachatus* and *Vipera* (Drummond & Yamamoto, 1971; Iwanaga & Suzuki, 1978) may belong to the e- N_s category. It is specific for nucleoside 5'-monophosphates and shows a preference for AMP. The enzyme from *Crotalus* has a molecular mass of about 60 kDa and contains zinc (Mannherz & Rohr, 1978; Fini *et al.*, 1990).

A soluble 5'-nucleotidase from human placenta was found to contain the same quantity of *myo*-inositol as the membrane anchored surface-located form (Klemens *et al.*, 1990). A soluble concanavalin A-binding 5'-nucleotidase from either electric ray electric organ or bovine brain is clearly derived from a GPI-anchored precursor by phospholipase C cleavage. This can be shown by antibodies (Hooper *et al.*, 1991) recognizing a specific epitope (inositol 1,2-cyclic monophosphate) of the cleaved anchor (Vogel *et al.*, 1992). In contrast, in rat kidney (Piec & Le Hir, 1991) at least part of the 'soluble' 5'-nucleotidase is hydrophobic and, after phase separation using Triton X-114, can be recovered in the aqueous phase only after treatment with phospholipase C. Similarly, the 'soluble' bull seminal plasma enzyme (in contrast to the human enzyme, Fini *et al.*, 1983, 1991) is presumably membrane-bound and can be extracted only with detergents. These forms presumably represent ectoenzyme residing on fragments of (shed) membranes (Trams *et al.*, 1981).

Taken together these results suggest that soluble 5'-nucleotidase e- N_s is derived from a GPI-anchored precursor (e- N) and thus is not truly cytoplasmic (Fig. 5). Presumably, the contribution e- N_s to fractions of other soluble 5'-nucleotidases might vary with the tissue source as well as the form of preparation and contribute to the kinetic data obtained with soluble extracts.

At present the source of the endogenous GPI-specific phospholipase is not entirely clear. Endogenous phospholipase C has been characterized in liver (Fox *et al.*, 1987; Stieger *et al.*, 1991) and phospholipase D (EC 3.1.4.4) in blood plasma (Low & Prasad, 1988). Endogenous phospholipases might release the enzyme under physiological conditions or else after excision of the tissue and on homogenization. The contribution to the total of this type of 5'-nucleotidase is generally low (1.5–6.8%) (Fritzson *et al.*, 1986; Piec & Le Hir, 1991). In bovine and rat brain a soluble low- K_m 5'-nucleotidase was, however, estimated to contribute more than 30% (Lai & Wong, 1991a) or even up to 94.5% (Montero & Fes, 1982) of total tissue enzyme activity. We find that starting from a contribution of about 30% the

amount of soluble (concanavalin A-binding) 5'-nucleotidase increases with *post mortem* time in bovine brain (Vogel *et al.*, 1992). The observation that the activity of soluble 5'-nucleotidase in synovial fluid varies with specific clinical symptoms (Wortmann *et al.*, 1991) or its presence in human seminal plasma (Fini *et al.*, 1991) and also snake venoms supports the notion that GPI-anchored 5'-nucleotidase may be released by a physiological mechanism. Whether this involves release from the surface or constitutive secretion remains to be elucidated. Examples for the release of GPI-anchored proteins include N-CAM, Thy-1 or the FcRIII-protein of neutrophils (Low, 1990).

Open questions. Not in all cases can the kinetic characteristics of soluble 5'-nucleotidases easily be grouped into a general scheme (see Newby, 1988; Skladanowski & Newby, 1990). There exist for example also reports on IMP-preferring enzymes with low K_m . Variations in kinetic data could be the result of species and/or tissue specific forms of the enzyme, analysis of impure preparations or alterations in kinetic parameters on enzyme solubilization and purification, and finally of variations in assay conditions (Dornand *et al.*, 1978; Panagia *et al.*, 1981; Montero & Fes, 1982; Harb *et al.*, 1983; Kaminska Berry *et al.*, 1986). For example, high concentrations of Mg^{2+} are able to reverse the inhibitory effect of nucleoside di- and triphosphates on soluble 5'-nucleotidase activity (Mallol & Bozal, 1983). Kinetic properties of soluble enzymes which apparently resemble both soluble and membrane-bound forms (Zekri *et al.*, 1988) might result from assay conditions which include high concentrations (10–20 mM) of $MgCl_2$ or $MgSO_4$.

BIOCHEMICAL PROPERTIES: BACTERIA

Bacteria contain both soluble and membrane-bound forms of 5'-nucleotidase encoded by different but homologous genes. Intrinsic membrane-bound 5'-nucleotidase is found in halophilic and marine bacteria of the genera *Vibrio* and *Photobacterium* (Table 3). At present the type of membrane anchorage (outer membrane) is unknown. The enzyme contains an *N*-terminal sequence very similar to the consensus sequence for the cleavage site of lipoproteins. Since the *N*-terminal residue of the mature enzyme is blocked, it seems to be likely that an acyl chain is attached (Tamao *et al.*, 1991). An apparent molecular mass of about 70 kDa has been estimated on SDS/PAGE and enzymes from various marine strains display immunological cross-reactivity (Bengis-Garber & Kushner, 1981; Itami *et al.*, 1989). Membrane-bound 5'-nucleotidase from *Vibrio* and *Photobacterium* can equally use nucleoside 5'-tri-, 5'-di-, and 5'-monophosphates as substrates with maximal activity at pH 8. 3'-Nucleotides are not hydrolysed. These membrane-bound and presumably outwardly oriented enzymes are unique in their requirement for Cl^- , particularly expressed in the purified enzyme. Mg^{2+} is required for activity which can partly be replaced by Mn^{2+} and Co^{2+} , whereas Zn^{2+} is inhibitory.

'Periplasmic' 5'-nucleotidase (UDP-sugar hydrolase) from *Bacillus subtilis* or from Enterobacteriaceae such as *Escherichia coli* is secreted into the periplasmic space and can be isolated as a soluble enzyme (Table 3). The apparent molecular mass is 52 kDa (Neu, 1967a). The periplasmic 5'-nucleotidase encoded by the *ushA* gene of *Escherichia coli* hardly deserves its name. This enzyme hydrolyses all 5'-ribo- and 5'-deoxyribonucleotides (including di- and triphosphates) with preference for AMP. 2'-, 3'-, or cyclic 2',3'-AMP are unaffected and the enzyme is not inhibited by phosphate (Glaser *et al.*, 1967; Neu, 1967a). In addition, this enzyme is also a uridine diphosphate sugar hydrolase, producing uridine monophosphate and glucose-1-phosphate. Nucleotide diphosphate sugars cleaved at essentially the same rate are UDP-D-glucose, UDP-D-galactose, UDP-N-

Table 4. Sources and properties of 5'-nucleotidase in plants

References are as follows: 1, Sharma *et al.* (1986); 2, Carter & Tipton (1986); 3, Mittal *et al.* (1988); 4, Ostergaard *et al.* (1991); 5, Polya & Ashton (1973); 6, Polya (1974, 1975); 7, Chen & Kristopeit (1981); 8, Carter & Tipton (1986); 9, Burch & Stuchbury (1986); 10, Sharma *et al.* (1986); 11, Mittal *et al.* (1988); 12, Ostergaard *et al.* (1991)

Source	Cellular location	Substrate	Requirement for metal ion	References
Corn microsomes, peanut cotyledons, soybean root nodules	Membrane-bound	AMP	(—)	[1–4]
Potato leaf and root, tomato plant, wheat germ, seedling, shoots and leaves	Soluble	All nucleoside 5'-monophosphates or only AMP; in some cases ADP, ATP or 3'-AMP	(—)	[5–12]

acetyl-D-glucosamine or UDP-N-acetyl-D-galactosamine. The enzyme shows an absolute requirement for a divalent cation. Like the vertebrate membrane-bound 5'-nucleotidase the bacterial soluble form appears to be a zinc metalloenzyme (Dvorak & Heppel, 1968). Apparent K_m values for AMP and also for UDP-D-glucose are in the low micromolar range (1–30 μM). The pH optimum for the hydrolysis for AMP is around 6 while that of UDP-glucose is between 7 and 8. Since the native molecular mass is about 137 kDa (Mauck & Glaser, 1970) the enzyme presumably exists as a dimer.

In addition to extracellular and membrane-bound 5'-nucleotidases, *Bacillus subtilis* has two cytoplasmic forms (Table 3). They differ in their activation by Mg^{2+} . The magnesium-requiring enzyme has a molecular mass of 23 kDa, a pH optimum of 7.5, and hydrolyses phosphomonoester bonds of purine and pyrimidine nucleoside 5'-monophosphates, but not UDP-glucose. It is strongly inhibited by Zn^{2+} , Cu^{2+} and Fe^{2+} and its K_m value for AMP is 0.25 mM.

BIOCHEMICAL PROPERTIES: YEAST

A 5'-nucleotidase from *Saccharomyces oviformis* shares with the bacterial *ushA* enzyme the property of hydrolysing all ribo- and deoxyribonucleoside 5'-phosphates but is incapable of hydrolysing sugar phosphates. It is unique in possessing nucleotide pyrophosphatase activity for hydrolysis of, e.g., NAD, $\text{NADH} + \text{H}^+$, FAD or ATP. It converts NAD⁺ first to AMP and the nicotinamide mononucleotide, followed by hydrolysis of AMP to adenosine and inorganic phosphate. Co^{2+} and Ni^{2+} act as activators. It has been suggested that the active sites for pyrophosphatase and nucleoside phosphatase activity reside on the same protein but at different sites (Takei *et al.*, 1969).

BIOCHEMICAL PROPERTIES: PLANTS

After the initial demonstration of 5'-nucleotidase in potato (see Drummond & Yamamoto, 1971) the further biochemical and molecular characterization of plant 5'-nucleotidase has received increasing attention in recent years. Both soluble and membrane-bound forms have been described (Table 4). Membrane-bound 5'-nucleotidase has been characterized in peanut cotyledons (Sharma *et al.* 1986; Mittal *et al.* 1988) and on SDS/PAGE displays a single band of 54–55 kDa. It is a glycoprotein (approx. 40% carbohydrate content) with a pH optimum between 5 and 6. There is no absolute metal ion requirement and the enzyme is highly specific for AMP. The cellular origin of enzymes from other sources is less defined. However, regarding their glycosylation and their source of isolation they may be membrane-derived. These include 5'-nucleotidase from corn microsomes (Carter & Tipton, 1986) and

soybean root nodules (Ostergaard *et al.*, 1991). The biochemical properties vary from source to source. No information is available concerning the type of membrane anchorage or membrane topography. 5'-Nucleotidase characterized in plant extracts after $(\text{NH}_4)_2\text{SO}_4$ precipitation might reflect soluble cytosolic forms. Sources include potato, wheat and tomato (Table 4). Native molecular masses vary between 50 and 69 kDa, but wheat germ contains an additional form of 110 kDa.

5'-Nucleotidase from various sources of higher plants varies considerably concerning functional properties. This can be due to species or tissues heterogeneity, as well as to the fact that preparations were only partly purified. Higher-plant 5'-nucleotidase appears to differ from the enzyme derived from other sources in being competitively inhibited by cyclic nucleotides (K_i in the low micromolar range) (Polya & Ashton, 1973; Polya, 1975; Carter & Tipton, 1986). An additional characteristic is their non-competitive inhibition by nucleotides. Generally, there is a preference for nucleoside 5'-monophosphates and AMP is the preferred substrate. There may also exist significant hydrolysis of 3'-AMP (Polya & Ashton, 1973; Polya, 1974, 1975; Carter & Tipton, 1986) or of ADP and ATP (Chen & Kristopeit, 1981). In contrast to animal enzymes, maximal activity at acidic pH (5–6) has been described for both membrane-bound and soluble forms. K_m values for AMP vary from millimolar to low micromolar values. If analysed, no absolute dependence on added divalent cations were found. Further studies are expected to reveal that plant 5'-nucleotidases, like the animal enzymes, can be divided into distinctive groups.

5'-NUCLEOTIDASE INHIBITORS

For both bacteria and plants, endogenous inhibitors of 5'-nucleotidase have been described. A 5'-nucleotidase-specific inhibitor is present in the cytoplasm of a number of bacteria including *Escherichia coli* (Dvorak *et al.*, 1966; Neu, 1967b). The inhibitor appears to be a proteinaceous substance as it is completely destroyed by pronase. It inhibits hydrolysis of 5'-AMP, ATP and uridine diphosphoglucose. A proteinaceous inhibitor specific for 5'-nucleotidase has also been described for lymphocytes (Sun *et al.*, 1983).

Polyphenolic substances which possess antitumour activity and inhibit 5'-nucleotidase from a variety of sources have been isolated from the seeds of *Areca catechu* (betel nuts) (Iwamoto *et al.*, 1988; Uchino *et al.*, 1988) as well as from wine grapes (Toukairin *et al.*, 1991). Since murine macrophages are directly stimulated by these inhibitors and no general cytotoxic effects are observed, the antitumor activity might be through potentiation of the immunity of host animals (Matsuo *et al.*, 1989). Since these 5'-nucleotidase inhibitors have also an inhibitory effect on

the growth of *Streptococcus mutans* they may be useful as dental plaque preventing agents (Iwamoto *et al.*, 1991).

GLYCOSYLATION

Both the membrane-bound form (e-N) of animal 5'-nucleotidase as well as the soluble form (e-N_s) that shares its general functional characteristics are glycosylated.

The analysis of cDNA-deduced primary structures reveals four (human placenta, electric ray brain) or five (rat liver) potential *N*-linked glycosylation sites. This corresponds closely to previous estimates of *N*-linked oligosaccharide chains using hepatic sources (five; van den Bosch *et al.*, 1986; Wada *et al.*, 1986) or human chorionic cells (four; Burgemeister *et al.*, 1990). By differential treatment with endoglycosidase H (which cleaves only high-mannose glycan chains) and endoglycosidase F (which cleaves both complex and high-mannose chains) the nature of the glycan chains has been further differentiated. In rat liver (Wada *et al.*, 1986; Baron & Luzio, 1987) all the glycan chains have been processed from the high-mannose type to the complex type. But studies from a rat hepatoma cell line (van den Bosch *et al.*, 1986) suggest a fraction of the 5'-nucleotidase keeps one or two high-mannose or hybrid chains in the mature form. There appears to be species- and tissue-specific variation, inferring hybrid type (Harb *et al.*, 1983; Burgemeister *et al.*, 1990), high-mannose type (Méfiah *et al.*, 1984a) or complex type (Méfiah *et al.*, 1984b) of glycosylation. There is no evidence yet for O-linked glycosylation.

Various chemical treatments proved that 5'-nucleotidase is a sialoglycoprotein (Harb *et al.*, 1983; Méfiah *et al.*, 1984a; Wada *et al.*, 1986, 1987; van den Bosch *et al.*, 1988; Buschette-Brambrink & Gutensohn, 1989). 5'-Nucleotidase from rat liver lysosomes (Wada *et al.*, 1987), human placenta (Buschette-Brambrink & Gutensohn, 1989), and also from electric ray electric organ (Vogel *et al.*, 1991b) can be separated by two-dimensional electrophoresis into up to 13 isoforms (pI ranges of 4.4–6.0, 5.8–7.0 and 5.9–6.7, respectively). Digestion of 5'-nucleotidase with neuraminidase reduces the number of spots towards alkaline forms (Wada *et al.*, 1987; van den Bosch *et al.*, 1988; Buschette-Brambrink & Gutensohn, 1989). The differences in pI may therefore be related to different contents in sialic acid residues in the isoforms. For comparison, the pI range for isoforms of soybean 5'-nucleotidase is completely different (7.5–8.5) (Ostergaard *et al.*, 1991).

These results extended earlier studies which first identified the glycoprotein nature of ecto-5'-nucleotidase using plant lectins (Riordan & Slavik, 1974; Carraway *et al.*, 1976, 1979). Lectins bind to 5'-nucleotidase and inhibit its activity to varying degrees depending on tissue and cell type. For example, concanavalin A inhibits the ecto-enzyme activity in all systems analysed so far. Wheat germ agglutinin inhibits enzyme activity in bovine hepatocytes and caudate nucleus, but not in lymphocytes. The lymphocyte enzyme does not appear to carry sialic acid (Méfiah *et al.*, 1984a,b). The notion of a heterogeneity in the carbohydrate moieties of 5'-nucleotidase from one cell type to another within the same species is further supported by immunochemical studies on the inhibitory effect of IgG-fractions in the presence of various sugars (Harb *et al.*, 1985). Primary structure analysis of carbohydrate chains should further clarify the degree of heterogeneity in 5'-nucleotidase glycosylation.

Recently, association of the HNK-1 epitope has been demonstrated for 5'-nucleotidase from the electric ray electric organ and cat brain (Vogel *et al.*, 1991a,b). The epitope recognized by the HNK-1 monoclonal antibody in glycoproteins was identified as glucuronic acid 3-sulphate. It is expected that the same or a very similar epitope is responsible for HNK-1 reactivity with 5'-nucleotidase. A number of glycoproteins such as N-CAM, L1, J1

or cytostatin which are involved in cell adhesion also carry this particular carbohydrate epitope.

BIOSYNTHESIS

Glycosylation of 5'-nucleotidase and processing of carbohydrate side chains were studied in cultured cells such as hepatoma cells (van den Bosch *et al.*, 1986), primary cultured hepatocytes (Baron & Luzio, 1987), and chorionic cells (Burgemeister *et al.*, 1990), as well as in cell-free systems from rat liver (Wada *et al.*, 1986; Misumi *et al.*, 1990a). These investigations indicate that the biosynthetic pathway of 5'-nucleotidase follows the general route taken by other membrane proteins carrying a GPI anchor. This involves its synthesis at the rough endoplasmic reticulum and co-translational cleavage of the signal peptide and core glycosylation. The addition of the GPI anchor is expected to occur in the lumen of the endoplasmic reticulum immediately after protein synthesis. The protein is then passed through the Golgi apparatus to the cell surface (van den Bosch *et al.*, 1986, 1988).

On translation *in vitro* an immunoprecipitable precursor form of 68–69 kDa can be identified in the microsomal fraction which is *N*-glycosylated and contains high-mannose oligosaccharide chains. This form appears to be rapidly passed to the Golgi apparatus where it will be converted to the mature form of 72–73 kDa by terminal glycosylation of its oligosaccharides. In rat hepatoma cells maturation proceeds with a halftime of approx. 25 min and the newly synthesized mature enzyme reaches the cell surface after 20–30 min (van den Bosch *et al.*, 1986). The general half-life of 5'-nucleotidase is about 30 h in rat hepatoma cells (van den Bosch *et al.*, 1986) and 23 h in cultured hepatocytes (Baron & Luzio, 1987). A similar value has been derived for trinitrobenzenesulphonic acid-inactivated glioblastoma cells (Salem & Trams, 1983). This is in the range expected for a protein with an *N*-terminal methionine, and is similar to that of other cell surface enzymes.

The mature form of the rat liver enzyme consists of 523 amino acid residues plus carbohydrates and the C-terminal GPI-anchor. Inhibition of *N*-glycosylation by tunicamycin yields a protein of 57–59 kDa (van den Bosch *et al.*, 1986; Wada *et al.*, 1986). This closely corresponds to the molecular mass expected for the unglycosylated protein. After loss of the signal peptide (3 kDa) and the 25 amino acids at the C-terminus (3 kDa), and after the addition of the GPI anchor (1.7 kDa) its mass would be 59.7 kDa. It is noteworthy that blockade of glycosylation or carbohydrate processing in human chorionic cells neither has an influence on the transfer of newly synthesized 5'-nucleotidase to the GPI anchor nor does it interfere with the transport of the enzyme to the cell surface (Burgemeister *et al.*, 1990).

CELLULAR DYNAMICS

After the ecto-enzyme nature of 5'-nucleotidase had finally been established the extensive intracellular distribution of the enzyme and the dynamic behaviour of its resident plasma membrane came as a surprise. The evidence was accumulated by subcellular fractionation, immunoelectron microscopy, and studies on tissue culture cells using pulse-chase labelling, inhibition of surface located enzyme activity, and manipulation of membrane recycling. Cellular systems that have been most intensively studied include hepatocytes, lymphocytes and adipocytes (see Luzio *et al.*, 1986, 1987) as well as fibroblasts and macrophages (see Thilo, 1985; Widnell *et al.*, 1986).

Varying with cell type, up to 50% of the enzyme resides intracellularly in a membrane-bound pool. Major contributors are an endocytotic pool including lysosomes (Maguire & Luzio, 1985; Wada *et al.*, 1987; Tanaka *et al.*, 1989) or also transcytotic

vesicles (Mullock *et al.*, 1983). Antibodies against ecto-5'-nucleotidase recognize the organelle-occluded enzyme. Using cytochemistry and immunoelectron microscopy this subcellular location, including labelling of the Golgi apparatus, can be confirmed (Farquhar *et al.*, 1974; Little & Widnell, 1975; Stanley *et al.*, 1983; Geuze *et al.*, 1984; van den Bosch *et al.*, 1988).

That 5'-nucleotidase in cultured hepatocytes (Stanley *et al.*, 1980), fibroblasts (Widnell *et al.*, 1982) or rat hepatoma cells (van den Bosch *et al.*, 1988) is continuously recycled from the cell surface to an intracellular pool and back can be shown by cell surface modulation. Application of inhibitory antibodies to the cell surface at 2 °C causes inhibition of most of the cell surface 5'-nucleotidase activity (Stanley *et al.*, 1980; Widnell *et al.*, 1982). On incubation at 37 °C the antibody starts to inhibit also intracellular 5'-nucleotidase (50 % after 30 min). Cells incubated with antibody at 2 °C, subsequently washed and kept at 37 °C recover surface-located enzyme activity but intracellular activity decreases. Since these processes are not sensitive to the inhibitor of protein synthesis cycloheximide, protein synthesis *de novo* is not involved in these phenomena. Similar results are obtained when ecto-5'-nucleotidase is inhibited with concanavalin A. They obviously reflect a high activity of membrane shuttle between extra- and intra-cellular pools. Further work will have to prove whether the extensive recycling of 5'-nucleotidase occurs preferentially in cultured cells or whether it is also a property of intact tissue.

At present it is not known whether endocytosis of the GPI-anchored 5'-nucleotidase involves coated pits or other endocytotic compartments. After previous surface-labelling with ¹²⁵I and removal of sialic acid residues with neuraminidase, cell surface-derived 5'-nucleotidase can be differentiated from intracellular 5'-nucleotidase (rat hepatoma cells, van den Bosch *et al.*, 1988). As with fibroblasts and hepatocytes the surface enzyme gradually disappears, reaching a steady state after 60–90 min with 48 % of the enzyme now being intracellular. If 5'-nucleotidase recycles together with surface-located receptors like the transferrin receptor the weak base primaquine (which neutralizes the pH of acidic intracellular membrane compartments such as endosomes and lysosomes) should equally block uptake of iron and the redistribution of 5'-nucleotidase. However, whereas primaquine blocks uptake of iron, recycling of 5'-nucleotidase is even accelerated. Possibly, 5'-nucleotidase can recycle via an additional pathway that is insensitive to weak bases and not accessible to the transferrin receptor.

The role of the lysosomal compartment in 5'-nucleotidase turnover has not yet been fully clarified. Lysosomes from rat liver contain both soluble (approx. 25 %) and membrane-bound (approx. 75 %) 5'-nucleotidase corresponding enzymically, immunologically, and also regarding their sialylation to the cell surface type (Maguire & Luzio, 1985; Wada *et al.*, 1987; Tanaka *et al.*, 1989). Phase separation experiments reveal that the soluble form is devoid of its GPI anchor whereas 70 % of the membrane-bound form can be released by phosphatidylinositol-specific phospholipase C. Possibly a lysosome-endogenous phosphatidylinositol-specific phospholipase C is responsible for the conversion from the membrane-bound to the soluble form.

CELLULAR AND SUBCELLULAR LOCATION

The cellular and subcellular location of 5'-nucleotidase can be most reliably documented by immunocytochemical techniques using either monoclonal or monospecific polyclonal antibodies. Most work is concerned with the localization of ecto-5'-nucleotidase. Such studies show that the distribution of the enzyme is not always homogenous, neither among the cellular elements of a given tissue nor at the surface of an individual cell.

Subcellular localization has been studied most intensively in liver tissue. Immunoelectron microscopical analyses of rat liver using antibodies directed against detergent soluble liver 5'-nucleotidase revealed both intracellular and surface located 5'-nucleotidase (Geuze *et al.*, 1984; Matsuura *et al.*, 1984). The density of 5'-nucleotidase is higher at the bile canalicular surface of hepatocytes than at the sinusoidal surface, where a microvillar location predominates. The lateral surface is essentially devoid of the enzyme. 5'-Nucleotidase is also found at the surface of the capillary endothelial cells and in hepatoma cells (van den Bosch *et al.*, 1988). Furthermore it is detected in coated pits, multivesicular endosomes, at the luminal faces of the Golgi membranes, in the trans-Golgi network, and with lipoprotein particles. These immunocytochemical studies are consistent with the high turnover of membrane-bound 5'-nucleotidase observed in hepatocytes. They confirm the cellular location revealed by conventional cytochemistry in liver (e.g. Farquhar *et al.*, 1974) and also in other cellular systems like rat fibroblasts (Widnell *et al.*, 1982), guinea pig neutrophils (Robinson & Karnovsky, 1983) or capillary endothelial cells (Nacimient & Kreutzberg, 1990).

An asymmetric distribution of 5'-nucleotidase has also been observed in other tissues. In rat kidney, the enzyme is situated at the brush border of proximal tubules, the apical cell membrane and the apical cytoplasm of intercalated cells in connecting tubules and collecting ducts (Le Hir & Kaissling, 1989; Le Hir *et al.*, 1989b; Gandhi *et al.*, 1990). It is important to point out that only part of the fibroblasts (the stellate shaped ones) and of the endothelial cells were immunoreactive. Likewise in other tissues like adrenal cortex, spleen and liver only an organotypic subset of capillaries is immunoreactive (Thompson *et al.*, 1990).

As well as being found at the surface of muscle cells (Heidemann *et al.*, 1985; Grondal *et al.*, 1988) the enzyme has also been immunolocalized in nervous tissue. In the peripheral nervous system 5'-nucleotidase is associated with the Schwann-cell membranes, including myelin (Grondal *et al.*, 1988). Using anti-(liver 5'-nucleotidase) antibodies the enzyme was detected in the central nervous system of rats at the surface of glial elements like Bergmann glia, astrocytic endfeet around blood vessels (cerebellum; Schoen *et al.*, 1987, 1988), oligodendroglia and myelinated fibres (Cammer *et al.*, 1985). This agrees with earlier cytochemical studies on various neuronal systems where a neuronal localization of 5'-nucleotidase was rarely observed (see Kreutzberg *et al.*, 1986, and more recently Dolapchieva *et al.*, 1988; Nacimient & Kreutzberg, 1990), and with studies on cultured glial cells (Lai & Wong, 1991b). However, functional investigations imply that in some brain regions 5'-nucleotidase is associated with neuronal surfaces like those of cholinergic nerve terminals from rat striatum (Richardson *et al.*, 1987). Apparently, 5'-nucleotidase is distributed heterogeneously between brain areas and even within the cerebral cortex (Nagata *et al.*, 1984; Hess & Hess, 1986; Eisenman & Hawkes, 1989).

The only immunocytochemical study using an antibody directed against cytosol 5'-nucleotidase (IMP-preferring type c-N-II) was performed on liver tissue. The antibody recognizes the cytoplasm in a variety of liver cells: parenchymal cells, Kupffer cells and endothelial cells (Yokota *et al.*, 1988). The immunolocalization demonstrates the general presence of the enzyme in the cytoplasmic matrix and its absence from cell organelles such as the endoplasmic reticulum, peroxisomes, or nuclei.

PHYSIOLOGICAL FUNCTIONS

Hydrolysis of nucleoside 5'-monophosphates at different evolutionary levels and within different cellular systems and pools serves varying physiological functions. The present review, which primarily deals with the molecular structure and biochemical

Fig. 6. General aspects of intracellular and extracellular nucleotide metabolism involving different 5'-nucleotidases

1, adenylyl kinase reaction; 2, adenylyl deaminase; 3, c-N-I form of 5'-nucleotidase; 4, c-N-II form of 5'-nucleotidase; 5 and 6, extracellular hydrolysis of ATP by activity of ecto-ATPase and ecto-ADPase or possibly also of ecto-nucleotide pyrophosphatase; 7, ecto-5'-nucleotidase (e-N); 8, adenosine kinase. At present it is uncertain whether c-N-I occurs in tissues other than heart. Both extracellular ATP and adenosine can activate specific receptors (red). Adenosine (or inosine) can be salvaged by reuptake into the cell via a specific nucleoside carrier and used for resynthesis of nucleotides. The extracellular space is tinted.

properties of 5'-nucleotidase, presents only a brief outline of general functional contexts involving the various 5'-nucleotidases.

Bacterial 5'-nucleotidase

In general, bacterial 5'-nucleotidases are multifunctional. Together with sugar 1-phosphatase (EC 3.1.3.23), periplasmic 5'-nucleotidases provide a complete system for the hydrolysis of an extracellular nucleoside diphosphate sugar (via the nucleoside monophosphate and sugar 1-phosphate) to nucleoside and nonphosphorylated sugar. For example:

Subsequently, the products are easily transported into the cell. Thus, periplasmic 5'-nucleotidase of bacteria such as *Bacillus* and the Enterobacteriaceae probably serves in providing a carbon source for the cell (Glaser *et al.*, 1967; Yagil & Beacham, 1975; Bengis-Garber & Kushner, 1981). The function of the intracellular 5'-nucleotidase inhibitor is not yet understood. It has been suggested that it prevents the enzyme from disrupting nucleic acid metabolism (Neu, 1967b).

Though the membrane-bound, surface-located 5'-nucleotidase of other bacteria (e.g. *Vibrio*, *Photobacterium*) does not hydrolyse nucleoside sugar diphosphates it is still capable of catalysing the complete hydrolysis of nucleoside triphosphates to adenosine. Therefore bacterial 5'-nucleotidase in both seawater and freshwater organisms can considerably contribute to the regeneration of phosphate (for uptake into phytoplankton) from dissolved organic phosphorous compounds (Ammerman & Azam, 1985; Cotner & Wetzel, 1991). Thus, bacterial 5'-nucleotidase has an ecological function in nutrient recycling in aqueous habitats.

Plant 5'-nucleotidase

That membrane-anchored 5'-nucleotidase in plants is a glycoprotein points to a potential ectolocation. But surface location has not yet been clearly established. The intracellular forms so far described have been implicated in the catabolic pathway leading to ureides (Christensen & Joachimsen, 1983), in cytokine metabolism (Chen & Christopeit, 1981; Burch & Stuchbury, 1986), and in the regulation of the nucleotide pool size (Carter & Tipton, 1986). Before potential individual forms of 5'-nucleotidases in plants, including their distribution in the various systematical groups, are identified more precisely general functional concepts are difficult to derive.

Animal 5'-nucleotidase

Surface-located 5'-nucleotidase. In animals the surface-located form of 5'-nucleotidase (e-N) is present in practically all tissues,

but not on all cell types. It has been implicated mainly in the extracellular hydrolysis of 5'-AMP to adenosine (Fig. 6). This can be demonstrated by extracellular application of AMP to tissue culture systems or subcellular fractions forming sealed compartments (synaptosomes from brain; Richardson *et al.*, 1987; Terrain *et al.*, 1989) or also by perfusion with AMP of tissues like heart, liver, skeletal muscle, and lung (see Gordon *et al.*, 1986). It should be noted also that alkaline phosphatase occurs in the form of a GPI-anchored surface protein (Hawrylak & Stinson, 1988). So far, however, detailed information is lacking concerning the relative tissue and cellular distribution of the two enzymes and their specific role in hydrolysing extracellular nucleotides.

It appears of considerable significance that enzyme activities for hydrolysis of ATP or ADP whose properties differ from known intracellular ATPases are associated with cell surfaces in many tissues including blood cells (Evans, 1974; Pearson, 1985; Gordon, 1986; Gutensohn & Rieger, 1986; Nagy, 1986; Grondal & Zimmermann, 1986, 1988; Zimmermann *et al.*, 1986). These enzymes resemble ecto-5'-nucleotidase with regard to their ability to hydrolyse purine and pyrimidine nucleotides at a comparable rate. The K_m value for ATP of ecto-ATPase is in the same range as that for the hydrolysis of AMP by ecto-5'-nucleotidase. So far, no mechanism for a cellular release of AMP has been described. 5'-Nucleotidase would thus represent the final enzymic step within a cascade that leads from the extracellular trinucleotide to the nucleoside. Reuptake of the nucleoside would then serve the salvage of released nucleotides (Arch & Newsholme, 1978; Stone, 1981).

However, additional functional aspects need to be considered. Since both ATP and adenosine induce receptor-mediated physiological functions, ecto-5'-nucleotidase is involved in two principal and related pathways, the inactivation and catabolism of ATP, and the formation of adenosine. ATP acts via purinergic (P_2) receptors (Burnstock, 1991) on a large variety of tissues including the cardiovascular system, non-vascular smooth muscle and skeletal muscle, and the nervous system (Stone, 1981; Slakey, 1985; Gordon, 1986). The extracellular inactivation of ATP represents a necessary step in its control as an intercellular mediator. The last step, catalysed by 5'-nucleotidase, in turn produces adenosine. Via specific receptors (P_1) (Ribeiro & Sebastiao, 1986; Linden *et al.*, 1991) adenosine induces a variety of physiological functions including vasodilation, a decrease in glomerular filtration rate, inhibition of renin release, inhibition of neurotransmitter release, inhibition of the immune and inflammatory response or lipolysis (Stone, 1981; Gordon, 1986; Savic *et al.*, 1990, 1991; Daval *et al.*, 1991). This does not imply that extracellular adenosine exclusively originates from extracellularly hydrolysed nucleotide. Adenosine might also be released by reversal of the plasma membrane carrier (see Fredholm & Hedquist, 1980; Stone, 1981; Newby, 1984). The cell- or tissue-specific physiological consequences of ATP-elimination and/or adenosine production vary and will not be discussed here. They have been subject of earlier reviews including their function in brain (Nagy, 1986), liver (Luzio *et al.*, 1986, 1987), vascular system (Pearson, 1986; Slakey *et al.*, 1986), lymphoid cells (Gutensohn & Rieger, 1986; Dornand *et al.*, 1986), and fibroblasts (Widnell *et al.*, 1986).

The source of extracellular ATP and other nucleotides is still debated. Cellular systems known to compartmentalize ATP in secretory vesicles are confined to cholinergic and adrenergic neurons, chromaffin cells (White *et al.*, 1987), and blood platelets. From these ATP (and ADP) can be released by controlled exocytosis and for each case the complete cascade of hydrolysis to adenosine has been demonstrated (Pearson *et al.*, 1980; Grondal & Zimmermann, 1986, 1988; Nagy, 1986; Richardson

& Brown, 1987; Terrian *et al.*, 1989; Torres *et al.*, 1990). Extracellular concentrations of nucleotides are expected to be in the millimolar range on focal release and are about 1 μ M in venous blood (Harkness *et al.*, 1984). Since a physiological mechanism for release of nucleotide has not yet been described for other cellular systems the almost ubiquitous presence of ecto-nucleotidases might appear enigmatic. Nevertheless, release of ATP without an apparent loss in viability has been demonstrated for a number of cellular systems such as working muscle, working heart, renal cortex, brain (Wu & Philis, 1978), vascular endothelial and smooth muscle cells (Pearson & Gordon, 1979), and further cellular systems (see Gordon, 1986; Grondal & Zimmermann, 1988). Non-specific mechanisms of nucleotide release from cells include hypoxia and trauma. Then rapid degradation and salvage of extracellular nucleotides is essential (Trams *et al.*, 1980). Since ecto-5'-nucleotidase is effectively inhibited by ATP and ADP, production of extracellular adenosine from AMP will be profoundly delayed on ATP release. This represents a physiological restraint of extracellular adenosine formation as long as higher concentrations of ATP (and ADP) are active in the interstitial space. Effective nucleotide concentrations may vary depending on the type of tissue (Gordon *et al.*, 1986, 1989).

Two well-documented cases exemplify the diversity of physiological functions where 5'-nucleotidase activity is involved. A fraction of cholinergic nerve terminals from rat striatum (but not from cerebral cortex) contains the complete set of enzymes for the hydrolysis of released ATP to adenosine. Activation of the nerve terminals results in release of acetylcholine and ATP. The extracellularly formed adenosine inhibits the release of more transmitter acetylcholine (and ATP) via specific (presynaptic) receptors. Anti-5'-nucleotidase antibodies which inhibit enzyme activity attenuate this effect. Thus, hydrolysis of AMP by ecto-5'-nucleotidase is an essential step in the adenosine-mediated autoinhibition of transmitter release (Richardson *et al.*, 1987). Reuptake of adenosine terminates its extracellular effect and also serves purine salvage.

The spiny lobster (*Panulirus argus*) is able to 'smell' AMP, ADP and also ATP. Interestingly, the chemosensory neurons and presumably surrounding cells of the olfactory organ (antennule) can dephosphorylate all these nucleotides. AMP degradation in this case effects the inactivation of the odorant. The 5'-nucleotidase involved in this process is inhibited by ADP and adenosine 5'-[α,β -methylene]diphosphate, as is the vertebrate ectoenzyme (Trapido-Rosenthal *et al.*, 1987, 1990; Gleeson *et al.*, 1991).

Cytoplasmic 5'-nucleotidases. To date, many studies have been performed on partly purified preparations. Since soluble cellular extracts contain additional phosphatases as well as kinases (e.g. Truong *et al.*, 1988; Sychala *et al.*, 1989; Yamazaki *et al.*, 1991) the physiological interpretation of the kinetic data obtained has yet to be viewed with some reservation.

The role of the two cytoplasmic 5'-nucleotidases is connected to intracellular degradation of ATP via AMP to adenosine or via IMP to inosine (Fig. 6). These enzymes are thought to be of particular functional importance in situations leading to lowered energy charge such as increased work load, anoxia or ischaemia. The AMP concentration within the cell needs to be under control to prevent free interconversion of AMP, ADP and ATP by adenylate kinase (EC 2.7.4.3) as well as excessive activation of glycogen mobilization and glycolysis. Since the products, adenosine or inosine, can leave the cell this may cause a release of nucleoside. The array of physiological processes affected by released adenosine is expected to be identical to that of extracellularly formed adenosine. It has been noted that released adenosine can have the function of a retaliatory metabolite and

eventually restrain or reverse the effects of intracellular ATP degradation (Newby, 1984; Skladanowski & Newby, 1990).

The substrate specificities of intracellular 5'-nucleotidases are still difficult to interpret. The IMP-preferring cytosolic form (c-N-II) appears to be present in all tissues although in varying concentrations (Itoh *et al.*, 1986; Itoh & Yamada, 1991). According to kinetic data presently available this enzyme would be capable of keeping intracellular levels of IMP in the low micromolar range and to respond to any increase in cytosolic AMP concentrations up into the millimolar range. Formation of inosine from IMP by c-N-II is preceded by deamination of AMP by adenylate deaminase (EC 3.5.4.6), an enzyme that, like c-N-II, is modulated by ATP (activator) and inorganic phosphate (inhibitor) (Spychala *et al.*, 1988; Spychala & Marszalek, 1991). Since kinetics for IMP-hydrolysis are hyperbolic but those for AMP hydrolysis sigmoidal, IMP is the preferred substrate of c-N-II (van den Berghe *et al.*, 1977; Itoh *et al.*, 1986) (Fig. 4). In the absence of ATP or ADP the K_m value for IMP is in the unfavourably low millimolar range. However, millimolar concentrations of ATP that are expected to be present in normal cells but also during hypoxia or mild ischaemia (Collinson *et al.*, 1987) shift the affinity for IMP into the low micromolar range (Itoh *et al.*, 1986; Truong *et al.*, 1988). Furthermore they increase several-fold the maximal enzyme activity. ATP stimulates also hydrolysis of AMP but the K_m value does not fall below 1 mM. This high K_m value is of advantage when AMP concentrations increase from resting concentrations (approx. 100 μ M) into the millimolar range as in the case of, e.g. ischaemia (Newby *et al.*, 1983, 1987).

The metabolic impact of enzyme activity might vary. It has been pointed out that in uricotelic animals such as chicken cytosolic dephosphorylation of IMP is the first step on the pathway of uric acid formation from IMP (Tsushima, 1986). In mammals a relation of the enzyme to DNA synthesis has been implied (Itoh & Yamada, 1991). Another possibility is that the production of inosine rather than of adenosine would avoid undesired physiological effects of adenosine. Until more is known about the relative preponderance of c-N-II and the variability between tissues in kinetic parameters it is difficult to predict whether it has a general role in cytosolic adenosine production under resting conditions (Worku & Newby, 1983; Newby, 1984; Itoh *et al.*, 1986).

The situation in heart appears to be different from that in other tissues. Here two cytosolic forms of 5'-nucleotidase have been described: c-N-I with a preference for AMP and c-N-II preferring IMP. In the absence of ADP the K_m -values for AMP of both AMP- and IMP-specific 5'-nucleotidase would be above the likely free cytoplasmic concentrations of AMP. But recent experiments suggest that micromolar concentrations of ADP shift the affinity for AMP selectively of c-N-I into the low micromolar range (Yamazaki *et al.*, 1991). Since this enzyme is not activated by ATP, enzyme activity would be controlled only by the free concentration of ADP. As a large proportion of ADP is bound to actin, free ADP concentrations are difficult to estimate (Lowenstein *et al.*, 1983). However, under conditions of lowered energy charge the cardiac free intracellular ADP concentration is expected to rise. This would cause activation of AMP hydrolysis by c-N-I. Furthermore, c-N-I is less susceptible to inhibition by P_i than is c-N-II. Possibly the heart can take advantage of two cytosolic AMP-hydrolysing enzymes: one with low K_m for low intracellular AMP concentrations and one with high K_m which becomes important when AMP concentrations rise to millimolar levels. Nevertheless, the physiological function of c-N-II in heart, which contains much lower adenylate deaminase activity than skeletal muscle, needs further elucidation (Truong *et al.*, 1988; Skladanowski & Newby, 1990).

At present a final picture of the relative role of the two intracellular 5'-nucleotidases in nucleoside formation at differing states of energy charge cannot yet be derived. It is possible that 5'-nucleotidase of the c-N-I type will also be discovered in tissues other than heart. A critical evaluation requires precise information concerning the true free concentrations of all factors that can potentially affect enzyme activity at the varying physiological states of the cell. Such factors include not only ATP, ADP, AMP, IMP and P_i but also inosine, glycerate 2,3-bisphosphate, diadenosine polyphosphates, pH and possibly factors yet to be uncovered.

Deficiency of soluble 5'-nucleotidase can cause disease. Congenital deficiency is associated with haemolytic anaemia. This is thought to result from an impairment in red blood cells of the hexose monophosphate shunt flux: activity of glucose-6-phosphate dehydrogenase is inhibited on increase of pyrimidine nucleotide concentrations resulting from the deficit in 5'-nucleotidase activity (Ramenghi *et al.*, 1991). The immune dysfunction in patients with adenosine deaminase deficiency might result from an increase in deoxyadenosine concentrations and the subsequent formation of cytotoxic dATP concentrations. Interestingly, B lymphoblasts as well as T lymphoblasts with increased cytosolic 5'-nucleotidase activity were found to be resistant to deoxyadenosine and presumably prevent the formation of cytotoxic dATP (Edwards *et al.*, 1979; Wortmann *et al.*, 1979; Carson *et al.*, 1991). Possibly, not 5'-nucleotidase but rather cytosolic deoxynucleotidase (EC 3.1.3.31; Höglund & Reichard, 1990; Fritzon, 1991) which prefers 5'-deoxyribonucleotides is mainly responsible for the hydrolysis of dAMP.

The observation that cytosolic 5'-nucleotidase is able to phosphorylate inosine and 2',3'-dideoxyribonucleosides has initiated studies on its potential participation in the metabolism of antiviral agents (Keller *et al.*, 1985; Johnson & Fridland, 1989; Carson *et al.*, 1991) and in the development of inhibitory substances (Skladanowski *et al.*, 1989).

PLASTICITY OF 5'-NUCLEOTIDASE

Maturation-dependent expression

In a number of cell types the expression of ecto-5'-nucleotidase is variable and appears to be regulated. Ecto-5'-nucleotidase is expressed or its expression is increased in cells during the process of maturation, migration or growth. This was documented for a variety of tissues and cellular systems (DePierre & Karnovsky, 1974a,b; Rodan *et al.*, 1977; Uusitalo & Karnovsky, 1977; Widnell *et al.*, 1982; Dornand *et al.*, 1986; Turnay *et al.*, 1989) as well as for numerous tumours (Stefanovic *et al.*, 1976; Carraway *et al.*, 1979; Mescher *et al.*, 1981; Amano *et al.*, 1983; Slakey, 1985; Haffner *et al.*, 1988; Krüger *et al.*, 1991).

The dynamic behaviour of ecto-5'-nucleotidase has been particularly intensively studied in lymphocyte differentiation and disease (Sunderman, 1990). The literature is only briefly summarized here. Enzyme expression depends highly on the physiological state of the cells. 5'-Nucleotidase is recognized as a maturation marker (differentiation antigen CD73) for both T and B lymphocytes. Mono- and poly-clonal antibodies raised by several groups reveal differential expression of the enzyme on subsets of cells (see Thompson *et al.*, 1990). Enzyme activity in peripheral T-cells is about ten times higher than in thymocytes (Edwards *et al.*, 1979). That of B-cells from adult peripheral blood is several-fold higher than that of fetal spleen and cord blood B-cells (Bastian *et al.*, 1984; Thompson *et al.*, 1986). Ecto-5'-nucleotidase-positive B-cells synthesize 8–26-fold more IgG per cell than cells that do not express the enzyme (Thompson & Ruedi, 1988). Consequently, ecto-5'-nucleotidase activity is

abnormally low in lymphocytes of patients with a variety of immunodeficiency diseases (see Thompson *et al.*, 1986, 1990; Meftah *et al.*, 1991). Low 5'-nucleotidase activity is also found in lymphocytes of patients with chronic lymphocytic leukaemia (Dornand *et al.*, 1982a, 1986) but can be enhanced in other cases (Fukunaga *et al.*, 1989; Pieters *et al.*, 1991). 5'-Nucleotidase deficiency presumably reflects incomplete lymphocyte maturation.

Control of expression

Cell surface expression of 5'-nucleotidase can be regulated by external factors. Retinoic acid or recombinant interferon- γ stimulate 5'-nucleotidase in blood monocytes (Murray *et al.*, 1988). Cultured rat glomerula mesangial cells reveal stimulated synthesis of ecto-5'-nucleotidase after application of macrophage-conditioned medium (Stefanovic *et al.*, 1989). The cytokines interleukin-1 β and tumour necrosis factor- α which are produced by activated macrophages mimic this effect in a dose-dependent manner. The activation in part depends on stimulation of prostaglandin E₂ production (Savic *et al.*, 1990). The intracellular agent mediating this response is cyclic AMP (Savic *et al.*, 1991). Future experiments will show whether the same or additional intracellular messengers cause 5'-nucleotidase activation in response to the various stimulators and in the various tissues.

5'-Nucleotidase-mediated signals

Expression of 5'-nucleotidase might be related to an increasing demand for extracellular nucleosides of the maturing cells. Lymphocytes have very low levels of purine biosynthesis *de novo* and are dependent on the purine salvage pathway (Dornand *et al.*, 1982b; Thompson, 1985; Shah *et al.*, 1986). Consequently, anti-5'-nucleotidase antibodies completely depress cell proliferation (Andre *et al.*, 1987). However, lymphocyte 5'-nucleotidase may not only serve metabolic functions. Human T lymphocytes can be induced to proliferate and to express interleukin-2 receptor and interleukin-2 secretion by application of poly- and monoclonal anti-5'-nucleotidase antibodies and submitogenic concentrations of phorbol myristate acetate, an activator of protein kinase C (Thompson *et al.*, 1989, 1990). In this respect these findings resemble experiments with antibodies directed against a number of other GPI-anchored proteins such as Thy-1, RT-6 or Ly-6 which likewise cause production of interleukin-2 and/or mitogenesis (Low, 1989). A hybridoma-cell-derived factor (B-cell anti-5'-nucleotidase, 'BAN') which is a protein of 44 kDa selectively inhibits production of IgG in mature B cells via 5'-nucleotidase (Johnson, 1985), and interferon- α is toxic only for human lymphoblastoid cells displaying high levels of 5'-nucleotidase (Johnson, 1991). Finally, activity of ecto-5'-nucleotidase is essential for the generation of alloreactive cytotoxic T lymphocytes (Massaia *et al.*, 1988). There is evidence that 5'-nucleotidase, like other surface-located and GPI-anchored proteins, is capable of transmitting activation signals to the cell interior (Thompson *et al.*, 1989; Massaia *et al.*, 1990; Saltiel, 1990). Although this appears to be an intriguing possibility, the nature of potential natural ligands needs further elucidation.

Dynamics in the nervous system

Interestingly, dynamic expression of 5'-nucleotidase is also found in nervous tissue. After injury of nervous tissue high activity of 5'-nucleotidase is observed with, for example, reactive cells such as microglial cells (rat facial nucleus; Kreutzberg & Barron, 1978), or Schwann cells, proliferating satellite cells and also fibroblasts (endoneuronal connective tissue, rat superior cervical ganglion; Nacimientos & Kreutzberg 1990). This implies an involvement of 5'-nucleotidase in regenerative processes within

nervous tissue. A distinct role of 5'-nucleotidase can also be anticipated for the postnatal maturation of mammalian brain. In the mature brain 5'-nucleotidase activity is found mainly in association with glial cells. In the developing rat cerebellum, however, it is also associated with the surface of migrating immature nerve cells (Schoen *et al.*, 1988) or even with a subset of synapses during part of their generation period (Bailly *et al.*, 1990; Schoen *et al.*, 1991). Even more intriguing is the observation that 5'-nucleotidase activity in the kitten visual cortex is selectively and transiently expressed during the development period which is characterized by the remodelling of ocular dominance territories (Schoen *et al.*, 1990). 5'-Nucleotidase might therefore have a function in activity-dependent modifications of cortical circuitry. As yet, a preliminary caveat appears necessary. These studies have been performed with classical enzyme histochemistry. It has been noted (Schoen *et al.*, 1988) that the anti-5'-nucleotidase antibody recognizes only a glial but never a neuronal localization of 5'-nucleotidase. It cannot be excluded that the observed neuronal expression of AMP-hydrolysing activity is due to other surface-located phosphatases.

A role in cell-cell and cell-matrix interaction

As with lymphocytes, increased expression of 5'-nucleotidase in the activated tissues could be explained by an increased demand of nucleoside whereby the cell activates the extracellular purine salvage pathway. But additional or even alternative possibilities need to be considered. Adenosine receptors are only partially colocalized with 5'-nucleotidase, at least in brain (Fastbom *et al.*, 1987). This contradicts a general functional connection of extracellular adenosine production (ecto-5'-nucleotidase activity) and adenosine function. Recent evidence points to the possibility that the highly glycosylated and sialylated ecto-5'-nucleotidase has functional properties other than its catalytic activity. In electric ray electric organ and cat cerebral cortex the enzyme can carry the HNK-1 sugar epitope which is associated with a variety of cell surface proteins implicated in cell-cell and cell-matrix interactions (Vogel *et al.*, 1991a,b). It has also been noted that the extracellular matrix proteins laminin and fibronectin modify 5'-nucleotidase activity from chicken gizzard smooth muscle (Dieckhoff *et al.*, 1986b). Binding of laminin and fibronectin to 5'-nucleotidase is inhibited by anti-5'-nucleotidase antibodies (Stochaj *et al.*, 1989a). 5'-Nucleotidase reconstituted into proteoliposomes binds fibronectin in a saturable and specific manner (Stochaj *et al.*, 1990). Furthermore, poly- and mono-clonal anti-5'-nucleotidase antibodies inhibit the spreading of chicken embryonic fibroblasts on a laminin (but not on a fibronectin) substratum (Codogno *et al.*, 1988). So far there is no evidence for the author's assumption that 5'-nucleotidase is a transmembrane protein and may thus bind to intracellular actin. Also other evidence that 5'-nucleotidase is associated with an actin-containing detergent insoluble matrix (Mescher *et al.*, 1981; Vedeler *et al.*, 1991) possibly has resulted from the limited solubilization of the GPI-anchored enzyme by Nonidet P-40 (Hooper & Turner, 1988). Nevertheless, the bulk of these results points to the possibility that ecto-5'-nucleotidase may play a role in the interaction of activated cells with either the extracellular matrix and/or with other cells. The soluble form of ecto-5'-nucleotidase (e-N₅) might be able to occupy distant binding sites, a potential function of soluble cell adhesion proteins too (Gower *et al.*, 1988).

COMPARISON WITH OTHER ENZYMES

A computer search suggests that there are no direct relations between ecto-5'-nucleotidase and other vertebrate enzymes on the basis of sequence identity. Vertebrate 5'-nucleotidase has

significant clusters of sequence identities with prokaryotic enzymes exhibiting 5'-nucleotidase, 3'-nucleotidase or phosphodiesterase activities. Similarly, vertebrate alkaline phosphatases share considerable sequence identity with *Escherichia coli* alkaline phosphatase (Kenny & Turner, 1987). Although there is little general sequence identity between *Escherichia coli* 5'-nucleotidase (*ushA*) and alkaline phosphatase their signal peptides share a five-amino-acid stretch in the hydrophobic region (Burns & Beacham, 1986b).

In spite of the lack of sequence identity there are intriguing biochemical and functional similarities between a number of vertebrate hydrolases. Although it is rather uncertain at present whether these reflect common ancestry or convergent evolution, some of these common properties are briefly listed. 5'-Nucleotidase, alkaline phosphatase, alkaline phosphodiesterase I and acetylcholinesterase have all GPI-anchored, cell-surface-located forms (see Low, 1990). At least acetylcholinesterase (Toutant & Massoulie, 1987; Chatonnet & Lockridge, 1989) and 5'-nucleotidase occur as soluble forms with the glycan attached to the solubilized molecules. The membrane-bound forms of acetylcholinesterase (Chatonnet & Lockridge, 1989), 5'-nucleotidase and alkaline phosphatase (Hawrylak & Stinson, 1988) have very similar molecular masses and occur as polymers of catalytic subunits, as disulphide-linked dimers and possibly also as tetramers. The phosphatases have in common their metalloprotein nature (presumably zinc binding). Both 5'-nucleotidase (Vogel *et al.*, 1991a,b) and acetylcholinesterase (Maily *et al.*, 1989) can carry the carbohydrate epitope HNK-1. Alkaline phosphatase (Burg & Feldbush, 1989), acetylcholinesterase (Layer, 1991) as well as 5'-nucleotidase are expressed at the surface of differentiating and proliferating cells including lymphocytes and nerve cells. It is possible that the function of each of these enzymes goes beyond simple enzyme activity. Structural relations have been noted between cholinesterases and non-catalytic proteins that seem to be involved in cellular interactions (Krejci *et al.*, 1991). Further information concerning both the functional and structural level, including the three-dimensional structure (Sussman *et al.*, 1991), will be helpful for interpreting these similarities more decisively. Possibly the number of vertebrate enzymes related to this context will even increase.

SUMMARY AND PERSPECTIVES

5'-Nucleotidase activity in vertebrate tissues reflects the activity of four different forms of enzymes, a surface-located GPI-anchored ecto-form (e-N), a soluble form derived from it by cleavage of the membrane anchor (e-N_s), and two cytosolic enzymes (c-N-I and c-N-II). Only the surface-located ecto-5'-nucleotidase (e-N) has been characterized in molecular terms. It is expected that future sequence work will reveal that the closely related cytoplasmic forms c-N-I and c-N-II represent different genomic products. As yet the possibility of tissue-specific forms of 5'-nucleotidase as well as of splicing variants cannot be excluded. The e-N form reveals significant sequence identities with multispecific nucleotidases in bacteria, indicating common origin. Multispecific 5'-nucleotidases also occur in yeast but plant enzymes generally display marked substrate specificity. A characterization of the three-dimensional structure and of the active site would provide a basis for the interpretation of substrate specificities.

The production of nucleosides is the general result of 5'-nucleotidase activity. But the physiological implication of this catalytic process at the cellular or tissue level varies. The intracellular enzymes c-N-I and c-N-II are involved in the

catabolism of AMP and IMP and also of other mononucleotides and correspondingly produce the nucleoside. They are thought to be of particular importance for the hydrolysis of AMP and IMP in situations of low energy charge. The stimulatory effect of ATP and/or ADP on both affinity and rate seems to be of major physiological significance. A detailed analysis of the general biochemical properties with highly purified enzyme preparations is essential for setting the functional interpretations on a reliable basis. Furthermore, any general functional interpretation of the cytosolic enzymes requires both intimate knowledge of other cytosolic enzymes capable of hydrolysing 5'-mononucleotides as well as of the actual free concentrations of the metabolites concerned.

The surface-located enzyme is involved in a number of functions which presumably do not mutually exclude each other. They rather supplement each other in their contribution to the physiology of cellular metabolism and tissue function. It is important to note that expression of ecto-5'-nucleotidase does not occur on all cells and can be developmentally regulated. Ecto-5'-nucleotidase is involved in the salvage of extracellular nucleotides. In addition, the enzyme has a major role in the control of tissue homeostasis. It effects the final step in the complete hydrolysis of the extracellular messenger ATP, and, at the same time, produces adenosine as a further extracellular messenger. But this polysialylated GPI-anchored metalloprotein might not only function as an enzyme. The increased expression of the protein at the surface of activated cells and during certain stages of cell maturation, its capability to bind fibronectin as well as the presence of the HNK-1 epitope in some tissues implies an involvement in cellular interactions such as cell recognition and cell adhesion or in cell-matrix relations. This calls for a detailed analysis also of the carbohydrate chains, including possible developmental changes in the glycosylation pattern. Studies with lymphocytes even imply a function in signal transfer from the cell surface to the interior. Possibly a new mechanism for activation of intracellular messengers via GPI-anchored proteins will evolve.

Finally, consensus should be sought on 5'-nucleotidase nomenclature. Since conventional enzyme nomenclature groups all forms described into one category the literature has become confusing. For example, at the bacterial level, periplasmic 5'-nucleotidase is multispecific and its relation to the vertebrate ecto-5'-nucleotidase can only be deduced from the primary structure. The cytosolic 5'-nucleotidases have kinetic properties and physiological functions completely different from those of the ectoenzyme and the soluble form derived from it. It is suggested that current enzyme nomenclature is extended by introducing subclasses of EC 3.1.3.5: 1 for c-N-I, 2 for c-N-II, and 3 for e-N and e-N_s. 5'-Nucleotidases from bacteria and plants could then be grouped according to their sequence identities with one of the vertebrate enzymes.

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