Structure–activity relationship of cytoplasmic 5′-nucleotidase substrate sites

Andrzej C. SKLADANOWSKI*‡, Carsten HOFFMANN†, Joachim KRASS†, Bernd JASTORFF† and Wiesław MAKAREWICZ*

*Department of Biochemistry, Medical University of Gdansk, ul. Debinki 1, 80-211 Gdansk, Poland, and †Institut für Organische Chemie, Abt. Bioorganische Chemie, Fachbereich 2, Universität Bremen, Loebecker Strasse, 28359 Bremen, Germany

Various 5′-nucleotidases (EC 3.1.3.5) exist in vertebrate tissues. The sequence and cDNA cloning of the membrane-bound *ecto*-5′-nucleotidase (e-N) and one of the cytosolic isoenzymes, IMP-preferring (c-N-II), but not the cytosolic AMP-preferring form (c-N-I), have been reported. While c-N-II has a broad tissue distribution, c-N-I is found only in vertebrate heart. The published data on substrate specificity involve mainly the naturally occurring nucleoside monophosphates, without a systematic structure–activity relationship study. In the present study we have used a series of AMP and IMP analogues to examine the structure–activity relationship for c-N-I and c-N-II in detail. The

rank order of activity of the test compounds differed substantially between c-N-I and c-N-II. c-N-I and c-N-II varied with respect to the following interactions with substrate: (1) hydrogen-bond formation with the substituent in the 6-position of the purine ring (a donor-type with c-N-I and an acceptor-type with c-N-II); and (2) hydrophobic attraction of the 6-position unsubstituted purine ring (more pronounced with c-N-I than with c-N-II). No better substrate than 5′-AMP was found for c-N-I. We propose that c-N-I functions as an AMP-binding protein in the myocardial cell with an important role during ischaemic ATP breakdown when AMP accumulates rapidly.

INTRODUCTION

5′-Nucleotidase (EC 3.1.3.5) is widely distributed in vertebrate tissues and has also been described in bacteria and plant cells. Different isoenzymes are found, either membrane-bound or in the cell cytoplasm. Their molecular structure and functional aspects have been reviewed recently [1], where Zimmermann proposed a classification of the isoenzymes according to their cellular location, molecular and kinetic properties: one membrane-bound form (*ecto*-5′-nucleotidase, e-N), one secreted (e-N_s), and two soluble cytoplasmic forms: c-N-I (AMP-preferring) and c-N-II (IMP-preferring). The metabolic and physiological roles of the different isoenzymes are often unclear. On the one hand they are part of the catabolic pathways of nucleotides, on the other, dephosphorylation of AMP yields the key regulatory signal molecule, adenosine, which interacts with second messenger metabolism via the different types of adenosine receptors [2]. Alteration of 5′-nucleotidase levels has been recognized in a considerable number of diseases [3].

While c-N-II has been identified in a number of vertebrate and invertebrate tissues, including heart [4,5], and has recently been cloned [6], c-N-I has to date been found only in vertebrate heart [7–11]. The apparent K_m values for AMP and IMP have been determined to be in the 100 μ M to the low millimolar range. Both isoenzymes are stimulated by adenosine phosphates. ADP, at micromolar concentrations, shifts the K_m value of c-N-I with AMP to the low millimolar range, but the hydrolysis of IMP by c-N-II is activated only at millimolar concentrations of either ATP or ADP. Structure–activity relationship studies with c-N-I and c-N-II have, so far, only been performed with 5′-nucleotide derivatives of the naturally occurring purine and pyrimidine bases. Reviewing these data [10–23], one cannot get a clear picture of which essential molecular interactions are responsible for the observed selectivities.

Isoenzymes, receptors and other binding proteins can be compared and characterized using a series of systematically modified analogues of the natural ligands. We have developed this approach for cAMP- and cGMP-binding proteins and were able to identify several characteristic types of molecular interactions ([24]; for review see also [25,26]).

There are several molecular differences between AMP and IMP that could account for the substrate selectivity of the c-N isoenzymes. Thus, they possess hydrogen-bond donor and acceptor potentials which are opposite at the 1- and 6-positions of their base moieties (adenine: 6-donor, 1-acceptor and hypoxanthine: 6-acceptor, 1-donor), although by tautomerization, 5′-IMP can offer the same hydrogen-bonding potential as 5′-AMP. Both purine bases are able to form charge-transfer (CT) complexes with aromatic amino acid side-chains but they differ with respect to their CT-acceptor potential. Both purine bases differ in overall dipole moment with respect to its value and direction. The adenine base is much more hydrophobic than hypoxanthine.

To investigate the factors underlying the nucleobase selectivity of c-N-I and c-N-II, and also to study their relative abilities to catabolize sugar- and phosphate-modified nucleotides, we have utilized a series of analogues selected for use according to the following rationale: (1) analogues with a changed hydrogen-bond potential in the base and the ribose moiety; (2) analogues with a changed ionic interaction potential of the phosphate moiety; (3) analogues with different CT potential and different hydrophobicity in the purine base moiety; (4) analogues changed to influence *syn*–*anti* conformation; (5) analogues changed with respect to chemical reactivity of the phosphate moiety; and (6) analogues with altered regioselectivity of the phosphate moiety.

The results show clear differences between the two isoenzymes in the mechanism of nucleobase recognition, but little difference in their pattern of reactivity with analogues where modifications were introduced to the sugar or phosphate moiety.

Abbreviations used: c-N-I, AMP-preferring 5′-nucleotidase; c-N-II, IMP-preferring 5′-nucleotidase; CT, charge transfer; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; MNDO, modified neglect of diatomic overlap.

‡ To whom correspondence should be addressed.

EXPERIMENTAL

Materials

[2-³H]AMP (47 MBq/mmol) was obtained from the Radiochemical Centre (Amersham, Bucks., U.K.). AMP (sodium salt), ADP (potassium salt) and ATP (sodium salt) were purchased from Boehringer-Mannheim (Germany). Sodium phenylphosphate was purchased from UCB (Brussels, Belgium) and ribose-5'-phosphate from Calbiochem-Boehringer (Mannheim, Germany). TDP, D,L-dithiothreitol, magnesium chloride, Hepes, concanavalin A, 2'-dAMP (compound 6; see Figure 1 and Table 1), 2'-dIMP, GMP (compound 9), XMP (compound 10), 6-mercaptapurine-ribofuranoside 5'-monophosphate (compound 11), 7-deaza-adenosine (tubercidine) 5'-monophosphate (compound 16), 1,N⁶-etheno-adenosine 5'-monophosphate (compound 15), 2'-AMP (compound 3), 3'-AMP (compound 4), adenosine 5'-monophosphorothioate (compound 2) and adenosine 5'-monophosphoromorpholidate (compound 5) were all purchased from Sigma (St. Louis, MO, U.S.A.). 8-Bromo-adenosine 5'-monophosphate (compound 17) was from ICN Chemicals. 8-Amino(aminoethyl)-adenosine 5'-monophosphate (compound 20) was purchased from Biolog-Life Science Institute (Bremen, Germany). 8-Hydroxy-AMP (compound 18), 8-amino(methyl)-AMP (compound 19) and 8-aza-AMP (compound 21) were gifts from Dr. J. P. Miller (SRI, Stanford, U.S.A.). The following analogues were synthesized in our laboratory according to the method of Genieser et al. [27] and purified by preparative HPLC: 1-NO-AMP (compound 7), 2-amino-purine-ribofuranoside 5'-monophosphate (compound 8), purine-ribofuranoside 5'-monophosphate (compound 13) and 6-S-methyl-mercaptapurine-ribofuranoside 5'-monophosphate

(compound 12). 7-Deaza-inosine 5'-monophosphate was produced by enzymic deamination of tubercidine 5'-monophosphate by use of rabbit muscle AMP deaminase (Sigma, St. Louis, MO, U.S.A.). The derivatives were identified by their characteristic UV spectra, fast atom bombardment (FAB)-MS and by NMR.

HPLC columns and imidazole were from Merck (Darmstadt, Germany).

Reagents used for the purification of the cytosolic 5'-nucleotidase isoenzymes were those described earlier [10].

Enzyme preparations

Cytoplasmic 5'-nucleotidase isoenzymes were isolated from rabbit hearts purchased from the local breeder. Enzyme purification was performed according to the procedure described by Yamazaki et al. [8] with some minor modifications [10]. In short, the procedure comprised (1) low-salt buffer homogenization, (2) centrifugation at 100000 g, (3) ammonium sulphate (284 g/l) precipitation while stirring and (4) phosphocellulose column chromatography using a gradient of 0–1.0 M NaCl. Enzyme activity during purification was assayed either by means of [2-³H]adenosine formation from [2-³H]AMP or phosphate liberation when 5'-IMP was used as substrate [28]. Two activity peaks could be separated at 0.3–0.4 and 0.6–0.7 M NaCl, which represented the cytosolic AMP-selective isoenzyme (c-N-I) with specific activity 450 m-units/mg of protein and the IMP-selective 5'-nucleotidase (c-N-II) exhibiting a specific activity of 1060 m-units/mg of protein respectively.

All enzyme preparations used were proven to be almost free of AMP deaminase (<10 m-units/mg of protein) and completely devoid of adenosine deaminase and phosphodiesterase. The

Figure 1 Structures of AMP analogues

Numbers in parentheses correspond to the numbering in Table 1.

Table 1 AMP derivatives used in this study

Retention times (t_R) of nucleotide products were measured on the reverse-phase HPLC system according to the method of Smolenski et al. [29] and are expressed in relation to an adenosine t_R of 11.90 min.

No.	Abbreviation	Name	Relative t_R (min)
1	5'-AMP	Adenosine 5'-monophosphate	1.00
2	5'-AMPS	Adenosine 5'-monophosphorothioate	1.00
3	2'-AMP	Adenosine 2'-monophosphate	1.00
4	3'-AMP	Adenosine 3'-monophosphate	1.00
5	5'-AMP-morpholidate	Adenosine 5'-monophosphoromorpholidate	1.00
6	2'-Deoxy-5'-AMP	2'-Deoxyadenosine 5'-monophosphate	1.01
7	1-NO-5'-AMP	Adenosine- N^1 -oxide 5'-monophosphate	0.76
8	2-NH ₂ -5'-PuMP	2-Amino-purine-ribofuranoside-5'-monophosphate	0.96
9	5'-GMP	Guanosine 5'-monophosphate	0.85
10	5'-XMP	Xanthosine 5'-monophosphate	0.80
11	6-SH-5'-PuMP	6-Mercaptopurine-ribofuranoside-5'-monophosphate	0.90
12	6-S-Me-5'-PuMP	(6-S-methyl)-mercaptopurine-ribofuranoside 5'-monophosphate	1.42
13	5'-PuMP	Purine-ribofuranoside 5'-monophosphate	0.95
14	5'-IMP	Inosine 5'-monophosphate	0.85
15	1,N ⁶ -etheno-5'-AMP	1,N ⁶ -etheno-adenosine 5'-monophosphate	1.05
16	7-CH-5'-AMP	7-Deaza-adenosine 5'-monophosphate	0.98
17	8-Br-5'-AMP	8-Bromoadenosine 5'-monophosphate	1.20
18	8-OH-5'-AMP	8-Hydroxyadenosine 5'-monophosphate	0.99
19	8-NH-Me-5'-AMP	8-Amino(methyl)-adenosine-5'-monophosphate	1.10
20	8-AEA-5'-AMP	8-Amino(aminoethyl)-adenosine 5'-monophosphate	0.96
21	8-N-5'-AMP	8-Aza-adenosine 5'-monophosphate	0.98

absence of secreted *ecto*-5'-nucleotidase in the preparations was checked by using concanavalin A (250 μ g/ml).

HPLC-based assay for the kinetics of hydrolysis of IMP and AMP analogues

The investigations of rabbit heart c-N-I and c-N-II specificity and the kinetics of hydrolysis were followed by means of HPLC. Incubations were performed in a total volume of 0.1 ml in Eppendorf tubes containing either 100 mM Hepes (pH 7.0), 30 mM NaCl and 10 mM MgCl₂ for c-N-I or 50 mM imidazole (pH 6.5), 500 mM NaCl and 50 mM MgCl₂ for c-N-II. Allosteric activators (1 mM ADP/0.01 mM TDP for c-N-I or 2.5 mM ATP for c-N-II respectively) and 5'-nucleotide analogues (0.5–20 mM) were added as indicated in Tables 2 and 4. Concentrations of compounds were determined by their absorption coefficients. The reactions were started by the addition of 40 μ l of the appropriately diluted enzyme [12.8 m-units (28.4 μ g) of c-N-I or 11 m-units (10.4 μ g) of c-N-II] and incubated for either 30 min or 16 h depending on the rapidity of the enzymic degradation.

Reactions were terminated by addition of 50 μ l of 1.6 M HClO₄. Protein was subsequently removed by centrifugation and the supernatants were neutralized with 10 μ l of 3 M K₃PO₄, centrifuged again and analysed by reverse-phase HPLC following the procedure described by Smolenski et al. [29]. The retention times of the riboside analogues were found to be within the range 9–17 min (see Table 1) according to their different lipophilicities.

One unit of activity is defined as the amount of enzyme producing 1 μ mol of nucleoside from the corresponding nucleoside monophosphate per min at 37 °C under the optimal condi-

tions for the respective isoenzyme. Protein was measured according to the method of Bradford [30].

Determination of non-specific phosphatase activity

The involvement of non-specific phosphatases in the dephosphorylation of nucleotides was checked by using 20 mM ribose-5-phosphate or 20 mM sodium phenylphosphate as substrates under the conditions adopted for measuring the activity of c-N-I or c-N-II (see above). After 16 h, the reaction was stopped by a 4 min incubation at 85 °C. The phosphate concentration was determined according to the method of Itaya and Ui [31].

The rates of production of free phosphate when ribose-5-phosphate was substituted for nucleoside-5'-monophosphates were found to be 1.3 % of the activity with 5'-AMP and < 0.1 % of the activity with 5'-IMP as a substrate for c-N-I and c-N-II respectively. The rate of dephosphorylation of ribose-5-phosphate by c-N-I isolated from pigeon heart was 6.9 % [10]. The rates of sodium phenylphosphate hydrolysis were < 0.1 % of the activities observed with nucleoside 5'-monophosphates. Thus we may exclude the involvement of non-specific phosphatases in the hydrolysis of purine nucleotides when measured under assay conditions that are optimal for c-N-I with 5'-AMP or c-N-II with 5'-IMP.

Determination of K_m and V_{max}

Kinetic constants were evaluated using the linear-regression method with a Hanes–Woelf transformation of the Michaelis–Menten equation. Checks were made beforehand to ensure a hyperbolic relationship between activity and substrate concentration.

Quantum mechanical calculations

Dipole vectors, highest-occupied-molecular-orbital (HOMO) and lowest-unoccupied-molecular-orbital (LUMO) energies of the heterocycles were calculated with the modified neglect of diatomic overlap (MNDO) method by using the program HyperChem® purchased from Hypercube Inc. (Waterloo, Ontario, Canada) based on the linear combination of atomic orbitals method of Dewar and Thiel [32].

RESULTS

Hydrolysis rate of AMP analogues

Twenty-one AMP derivatives (Table 1, Figure 1) were studied. We ranked the biological activity of the analogues into four categories: 100–80 % (A), 55–25 % (B), 18–2 % (C) and less than 1 % (D), according to the hydrolysis rate measured with the best substrates (5'-AMP for c-N-I and 5'-IMP for c-N-II), as shown in Table 2.

No compounds with an identical ranking for both isoenzymes were found in category A. In category B, 6-Me-S-5'-PuMP (12) and 5'-XMP (10) exhibited a comparable activity with c-N-I and c-N-II. In category C, this was observed for 6-SH-5'-PuMP (11) and 8-N-5'-AMP (21). Almost identical behaviour with respect to the two isoenzymes was observed for those compounds modified within the phosphate moiety [2'-AMP (3), 3'-AMP (4), 5'-AMPS (2) and 5'-AMP-morpholidate (5)]. Additionally, 8-AEA-5'-AMP (20), carrying a positively charged side-chain at the 8-position, was found to be resistant to hydrolysis by either

Table 2 Hydrolysis of AMP analogues by the cytoplasmic 5'-nucleotidases c-N-I and c-N-II

Incubations were performed as described in the Experimental section with 5 mM analogue and 1 mM ADP or 2.5 mM ATP as an activator for AMP- (c-N-I) or IMP-selective (c-N-II) isoenzymes respectively. Reactions with compounds in categories A and B were analysed after 30 min and with compounds in categories C and D after 16 h. Means of duplicate estimations are presented.

Category (range of activity)	Analogues hydrolysed by 5'-nucleotidase	
	AMP-selective (c-N-I)	IMP-selective (c-N-II)
A (8–6 µmol/30 min per mg of protein)	5'-AMP	5'-GMP
	8-OH-5'-AMP	5'-IMP
	2'-Deoxy-5'-AMP	
	7-CH-5'-AMP	
B (4–2 µmol/30 min per mg of protein)	6-Me-S-5'-PuMP	6-Me-S-5'-PuMP
	8-Br-5'-AMP	5'-XMP
	2-NH ₂ -5'-PuMP	7-CH-5'-AMP
	5'-GMP	
	5'-IMP	
	1, N ⁶ -etheno-5'-AMP	
	5'-XMP	
	1-NO-5'-AMP	
	5'-PuMP	
C (45–3 µmol/16 h per mg of protein)	8-NH-Me-5'-AMP	5'-AMP
	6-SH-5'-PuMP	1, N ⁶ -etheno-5'-AMP
	8-N-5'-AMP	8-OH-5'-AMP
		2'-Deoxy-5'-AMP
		1-NO-5'-AMP
		8-Br-5'-AMP
		6-SH-5'-PuMP
		8-N-5'-AMP
D (< 3 µmol/16 h per mg of protein)	5'-AMP-morpholidate	5'-PuMP
	5'-AMPS	8-NH-Me-5'-AMP
	2'-AMP	2-NH ₂ -5'-PuMP
	3'-AMP	5'-AMP-morpholidate
	8-AEA-5'-AMP	8-AEA-5'-AMP
		5'-AMPS
		2'-AMP
		3'-AMP

Table 3 Comparison of c-N-I with c-N-II forms of rabbit heart cytosolic 5'-nucleotidase

Values are those recalculated from the experiment illustrated in Table 2 and they are means of two replicates ± S.E.M.

Substrate	Specific activity (units/mg of protein)	
	c-N-I	c-N-II
5'-AMP (1)	0.235 ± 0.028	0.047 ± 0.003
5'-IMP (14)	0.103 ± 0.011	0.247 ± 0.025
5'-GMP (9)	0.105 ± 0.012	0.254 ± 0.007
5'-XMP (10)	0.068 ± 0.009	0.114 ± 0.014
6-SH-5'-PuMP (11)	0.006 ± 0.001	0.007 ± 0.001
6-Me-S-5'-PuMP (12)	0.134 ± 0.019	0.132 ± 0.016
5'-PuMP (13)	0.056 ± 0.008	0.006 ± 0.001
2-NH ₂ -5'-PuMP (8)	0.111 ± 0.017	0.004 ± 0.001

isoenzyme. Most compounds were found in category B for c-N-I but in category C for c-N-II.

In Table 3, the hydrolytic activities of c-N-I and c-N-II isoenzymes of 5'-nucleotidase from rabbit heart are compared

Table 4 Specificity of different isozymes of rabbit heart 5'-nucleotidase relative to AMP

K'_m is the apparent K_m of the analogue relative to AMP; $K'_m = K_m(\text{analogue})/K_m(\text{AMP})$. $V'_{\max}$ is the maximum velocity of the hydrolysis of the analogue relative to AMP; $V'_{\max} = V_{\max}(\text{analogue})/V_{\max}(\text{AMP})$. Reactions were followed in duplicates for 30 min with six different concentrations of analogue (0.5–20 mM) and 0.01 mM 2'-deoxy-thymidine-5'-diphosphate or 2.5 mM ATP as activators for c-N-I and c-N-II respectively. The linearity of product accumulation during 30 min incubations was checked with 5'-AMP (1) and 2'-deoxy-5'-AMP (6) as substrates for c-N-I and with 5'-IMP for c-N-II. The correlation coefficients for the Hanes–Woolf linearization of the Michaelis–Menten equation for different analogues were always higher than 0.820. *Values for chicken heart IMP-selective 5'-nucleotidase (from [16]).

Analogue	5'-Nucleotidase isoenzyme			
	c-N-I		c-N-II	
	K'_m	$V'_{\max}$	K'_m	$V'_{\max}$
5'-AMP (1)	1	1	1	1
2'-dAMP (6)	0.13	0.06	2.62*	0.66*
5'-IMP (14)	1.00	0.23	0.15	2.22
5'-GMP (9)	0.73	0.17	0.39	3.65
5'-XMP (10)	1.64	0.38	0.29	1.64
6-Me-S-5'-PuMP (12)	1.64	0.38	0.23	2.26

for a set of eight characteristic analogues. The data clearly indicate that the activity profiles of the two isoenzymes are very different. There are only two exceptions: 6-SH-5'-PuMP (11) and 6-Me-S-5'-PuMP (12). The latter compounds have identical reactivity with each isoenzyme, being either relatively good (compound 12) or poor (compound 11) substrates for both isoenzymes. The AMP-selective isoenzyme (c-N-I) clearly differentiates between 5'-AMP (1) and 5'-IMP (14) or 5'-GMP (9), while the so-called IMP-selective form (c-N-II) does not differentiate between 5'-IMP (14) and 5'-GMP (9), but only 5'-AMP (1). 5'-PuMP (13) and 2-NH₂-5'-PuMP (8) are much better substrates for c-N-I than for c-N-II.

Kinetic analysis of the hydrolysis of selected analogues

For detailed kinetic study, we used the naturally occurring 5'-nucleotide derivatives (compounds 1, 6, 9, 10, 14) and additionally we selected the synthetic analogue 6-Me-S-5'-PuMP (12), which was ranked identically in category B for both isoenzymes.

Table 4 shows values for the kinetic constants K_m and $V_{\max}$ (K'_m and $V'_{\max}$) expressed in relation to those for 5'-AMP with c-N-I ($K_m = 8.3$ mM, $V_{\max} = 91.2$ units/mg of protein) or c-N-II ($K_m = 18.4$ mM, $V_{\max} = 22.6$ units/mg of protein). Interestingly, 2'-dAMP (6) exhibited the highest affinity but the lowest maximum velocity with the c-N-I isoenzyme. The same difference between binding and hydrolysis rate was obtained for 5'-GMP (9), while 5'-IMP (14), 5'-XMP (10) and 6-Me-S-5'-PuMP (12) were less well bound but relatively better hydrolysed.

In contrast, 5'-IMP (14) had the highest affinity for c-N-II and was also hydrolysed faster than 5'-AMP (1). The same was true for 5'-GMP (9), 5'-XMP (10) and even for the most hydrophobic analogue, 6-Me-S-5'-PuMP (12). On the other hand, 2'-deoxy-5'-AMP (6) was less well recognized and bound compared with 5'-AMP (1) and was also hydrolysed more slowly.

The polarity of the compounds (as indicated by their retention times; Table 1) did not correlate with either binding or rate of hydrolysis.

DISCUSSION

Phosphate moiety modifications

Hydrolysis by either c-N-I or c-N-II isoenzymes was drastically reduced through modifications introduced into the phosphate moiety. Neither the exchange of one negatively charged oxygen atom by sulphur [5'-AMPS, (2)] nor the substitution of that oxygen by a secondary amine [5'-AMP-morpholidate, (5)] is allowed. This clearly indicates that the monophosphate moiety is not only bound by a charge-charge interaction, which can still be formed easily by both derivatives, but that this interaction has to be supported by hydrogen-bonding donated either by the protein or by the protonated monophosphate moiety. The relative resistance against hydrolysis of 5'-AMPS presumably reflects the fact that nucleophilic attack at phosphorus is strongly decreased in phosphorothioate esters compared with phosphate esters [33,34].

The very poor hydrolysis of 2'-AMP (3) and 3'-AMP (4) confirms earlier results that both 5'-nucleotidases are absolutely regioselective for 5'-phosphoester substitution [11,21]. Since all stereochemical and chemical modifications at the phosphate moiety investigated in this study induced resistance to hydrolysis by either isoenzyme, one can conclude that the binding domain for the phosphate moiety of both isoenzymes must be very similar.

Ribose moiety modification

The analogue 2'-dAMP (6) was hydrolysed by c-N-I with higher affinity but with a lower $V_{\max}$ value than AMP. Lower affinity for 2'-dAMP compared with AMP but an unchanged $V_{\max}$ value is reported for c-N-II from chicken heart ([16]; Table 4). In our hands, 5'-AMP and its 2'-deoxy derivative showed similar behaviour towards c-N-II.

Thus the 2'-OH group is not essential for binding and enzymic catalysis for either isoenzyme. We can also conclude that the 2'-OH function does not participate in regioselective binding of nucleoside monophosphate.

Nucleobase moiety modifications

The c-N-I isoenzyme hydrolysed all the tested nucleobase-modified analogues apart from 8-AEA-5'-AMP (20), which has an additional positive charge at physiological pH on the β -amino group of the substituent. This offers the possibility of forming an intramolecular ion-pair between the negatively charged phosphate moiety and the positively charged amino substituent. Model building and computer modelling (results not shown) revealed that such a secondary structure can easily be formed without steric hindrance. This consequently changes the conformation of the phosphate moiety and we propose that this is mainly responsible for the decrease in biological activity. In parallel, the reduction of the negative charge-density of phosphate by the betaine structure will influence the mechanism of binding. Similar behaviour of c-N-II with AMP analogues was observed. Additionally, 8-NH-Me-5'-AMP (19) and 2-NH₂-5'-PuMP (8) were not dephosphorylated.

Four other test compounds (17–19, 21), besides the mentioned 8-AEA-5'-AMP (20) derivative, were modified at position 8 of the base moiety. The effects of these modifications were different. While the 8-OH substitution, which also has to be called 8-oxo substitution due to tautomerism, does not interfere strongly with binding and catalysis, the substitution of C-8 by nitrogen does reduce the biological activity substantially. Model building indicates that these two modifications should not influence the *syn-anti* equilibrium which for either 5'-AMP or 5'-GMP is

Table 5 Theoretically determined physicochemical properties of the bases of investigated nucleotides

Size and direction of the dipole-moments relative to the plane of the aromatic ring (the dipole moment of adenine is set to 0 °), and the HOMO/LUMO values were calculated by quantum mechanics as described in the Experimental section. n.d., not determined

Compound	Dipole moment		HOMO (eV)	LUMO (eV)
	Size (Debye)	Direction (°)		
Adenine	2.11	0.0	−8.81	−0.11
8-OH-Adenine	2.23	319.0	−8.64	−0.13
7-CH-Adenine	3.04	28.4	−8.43	0.10
6-Me-S-Purine	4.32	333.6	−9.11	−0.70
8-Br-Adenine	1.12	358.1	−8.95	−0.36
2-NH ₂ -Purine	2.86	280.1	−8.69	−0.17
Guanine	5.98	244.6	−8.52	−0.07
Hypoxanthine	4.89	262.4	−8.98	−0.45
1,N ⁶ -etheno-Adenine	4.60	254.9	−8.62	−0.61
Xanthine	6.84	298.0	−9.25	−0.22
Purine	3.37	319.3	−9.53	−0.55
6-SH-Purine	2.95	304.0	−9.16	−0.70
8-NH-Me-Adenine	2.55	n.d.	−8.99	−0.37
8-N-Adenine	0.75	327.0	−9.46	−0.54
1-NO-Adenine	5.07	342.0	−8.12	−0.68

reported as 0.3:0.7 [35]. The reason why they affect the biological activity differently is unknown.

Model building demonstrates also that 8-bromo- (compound 17) and 8-amino(methyl)- (compound 19) substituted AMP both favour the *syn*- over the *anti*- conformation. Since they are hydrolysed only relatively well by c-N-I, we suggest that the c-N-I isoenzyme prefers 5'-AMP in *anti*- conformation when recognized and bound by the enzyme. Our experience with 8-modified cAMP-derivatives [24] supports this conclusion because 8-bromo and 8-amino(methyl) modifications resulted in a reduction of affinity and thus enzymic activity in *anti*-type cAMP receptors (catabolite gene activator-protein from *Escherichia coli* and cell-surface receptors from *Dictyostelium discoideum*) but an increase in affinity for *syn*-type receptors (cAMP-dependent protein kinase type). At this moment we cannot comment on c-N-II preferences for *syn*- or *anti*-conformations as no 8-derivative of 5'-IMP was used.

Substituting the hydrogen-bond acceptor potential of adenine by the hydrophobic CH- group (compound 16) did not substantially inhibit binding or catalysis by either c-N-I or c-N-II, suggesting that neither isoenzyme binds adenine via hydrogen-bonding to N-7. This is in contrast with data obtained with AMP deaminase (A. C. Skladanowski, unpublished work) and adenosine deaminase [36]. In both deaminases, the 7-nitrogen atom is essential for biological activity, however, the 7-nitrogen was found not to be essential for cAMP binding to protein kinases [37].

The data obtained for derivatives with modified or substituted 6-amino function in the adenine base (9, 11–15) did not yield inactive compounds but only marginally influenced biological activity against c-N-I, with the exception of 6-SH-5'-PuMP (11) which was in category C for both isoenzymes. Therefore we conclude that no essential hydrogen-bonding between the 6-amino group and the enzyme is required to obtain catalytic activity, and c-N-I is thus only selective for 5'-AMP but is not specific. On the other hand, c-N-II clearly can differentiate adenine from guanine, hypoxanthine or xanthine bases (Table 3).

The strongest change in molecular interaction potential at the

6-position is introduced by the 6-methylthio substituent which is rather hydrophobic and offers no hydrogen-bond donor-acceptor potential at all. Nevertheless, this compound exhibited relatively high biological activity with both isoenzymes. That additional hydrophobic interaction is offered by the nucleobase at the binding domain is well accepted. This conclusion is supported by the behaviour of 1,*N*⁶-etheno-5'-AMP (15), 5'-PuMP (13) or 2-NH₂-5'-PuMP (8), which were all found to be in category B for c-N-I (Table 2). However, the last two compounds were one (13) or two (8) categories less good substrates for c-N-II than for c-N-I, which means that not only hydrophobic interactions but also dipole-dipole ones might be important in c-N-II. At the moment we can offer no rational explanation as to why the very polar hypoxanthine [5'-IMP (14)], xanthine [5'-XMP (10)] or guanine [5'-GMP (9)] bases are bound as well as the very hydrophobic 6-Me-S-5'-PuMP (12). In summary, a lack of binding interactions in both forms of the enzyme at the 6-position is likely, but we need to confirm this with use of the next generation of analogues.

Since none of the substitutions at N-1 (compounds 7 and 15) prevents binding and hydrolysis, we conclude that the electron pair on N-1 is not essentially involved in hydrogen-bonding with c-N-I. This is in contrast with the data obtained for the c-N-II isoenzyme, where it seems to be one of the interactions making this enzyme selective for 5'-GMP and 5'-IMP.

Polarizability and polarization

All modifications introduced to the base of 5'-AMP influence the potential for polarization or polarizability of the π -electron system. Semi-empirical calculations (MNDO) reveal this effect by means of different values for the HOMO and LUMO (Table 5). It seems rational to conclude that the adenine base of 5'-AMP is bound to c-N-I by CT interactions offered by an aromatic side-chain within the protein, since no essential hydrogen-bonding between the base and the protein could be found. Hydrophobic CT interactions could bind either an adenine or hypoxanthine/guanine moiety to the substrate-binding site with different affinities.

In addition, another differentiation between adenine and hypoxanthine or guanine bases is the value and the direction of the total dipole moment of the base moiety. Comparison of the nucleobases in the best four substrates for both isoenzymes (categories A and B) revealed that the mean size of the dipole moment is two times higher and turned approx. 45° anticlockwise for 5'-GMP, 5'-IMP, 5'-XMP and 6-Me-S-5'-PuMP (c-N-II substrates) compared with 5'-AMP, 8-OH-5'-AMP, 7-CH-5'-AMP and 6-Me-S-5'-PuMP (c-N-I substrates).

This difference may very well account for the selectivity of c-N-I towards adenine and c-N-II towards guanine and hypoxanthine due to dipole-dipole or dipole-induced dipole interaction. Thus we suggest that a yet undefined protein dipole might be responsible for base recognition and binding. X-ray analysis of the binding domains of the adenine moiety in other proteins revealed that for unselective binding a phenylalanine side-chain [38] is responsible, whereas for selective binding tyrosine or tryptophan moieties are responsible [39]. Phenylalanine binds the purine base via a dipole-induced dipole while tyrosine and tryptophan binding is based on a dipole-dipole interaction. CT interactions can be, but may not necessarily be, involved additionally.

Metabolic significance of activity of AMP-selective 5'-nucleotidase

Limited information is available on the species and organ distribution of the AMP-selective 5'-nucleotidase. Heart is the

only organ where this isoenzyme has been found so far. Compared with 5'-AMP, other naturally occurring nucleotides, such as, 2'-deoxy-5'-AMP, 5'-GMP, 2'-deoxy-5'-GMP, 5'-IMP, 2'-deoxy-5'-IMP, 5'-UMP and 5'-CMP, show a 1.5–3-fold lower rate of dephosphorylation. 5'-TMP and 2'-deoxy-5'-CMP are hydrolysed even 7–8 times more slowly by the AMP-selective 5'-nucleotidase (c-N-I) from pigeon [10] or dog [11] heart. Pronounced activity of c-N-I, especially in pigeon heart [40], contrasts with a relatively high K_m resulting in a low affinity for AMP, yet physiological concentrations of unbound AMP in myocardial cytoplasm are reported not to exceed 10⁻⁶ mol/l [41].

Formation of adenosine, which can protect underperfused, oxygen deprived heart and 'retaliate' against further stimulation [42], is well established but is perhaps not the only function of the c-N-I isoenzyme. The other physiological role for this isoenzyme, which in most predictable situations seems to be at a low saturation by AMP, may be the formation of an efficient AMP-overflow safety system in the cardiomyocyte when ATP degradation is very fast. The concentration of AMP within the cell needs to be under strict control to prevent free interconversion of adenine nucleotides by adenylate kinase as well as excessive activation of glycogen mobilization. As the adenylate kinase reaction is shifted to ATP production in ischaemic heart, a system keeping 5'-AMP and the other monophosphate nucleosides at a low level must exist. AMP deaminase competes for the same substrate and produces IMP (for review see [43]). The latter nucleotide might be saved for adenine nucleotide pool restoration when oxygen delivery is returned to normal. It may also be further degraded by the coexisting c-N-II, giving inosine. Thus the bulk of the AMP accumulating is removed from the cell as adenosine and inosine, or retained as IMP.

The results are consistent with the hypothesis that c-N-I might fulfil the role of a buffer for AMP, particularly when the environment favours higher local concentrations comparable to the Michaelis constant of this isoenzyme.

This study was supported by the Polish Committee for Scientific Research with grant 4 4034 91 02 (A.C.S.). Support from DAAD (Deutsche Akademischer Austauschdienst) for J. K. and C. H. during a visit to Gdansk and for A. C. S. in Bremen is appreciated. B. J. was supported by Fonds der Chemisches Industrie and Deutsche Forschungsgemeinschaft (Ja 246/6).

REFERENCES

- Zimmermann, H. (1993) *Biochem. J.* **285**, 345–365.
- Olsson, R. A. and Pearson, J. D. (1990) *Physiol. Rev.* **70**, 761–845.
- Sunderman, F. W., Jr. (1990) *Ann. Clin. Lab. Sci.* **20**, 123–139.
- Skladanowski, A. C. and Newby, A. C. (1991) in *Role of Adenosine and Adenine Nucleotides in Biological Systems* (Imai, S. and Nakazawa, M., eds.), pp. 289–299, Elsevier, Amsterdam, New York, Oxford.
- Itoh, R. (1993) *Comp. Biochem. Physiol.* **105B**, 13–19.
- Oka, J., Matsumoto, A., Hosokawa, Y. and Inoue, S. (1994) *Biochem. Biophys. Res. Commun.* **205**, 917–922.
- Truong, V. L., Collinson, A. R. and Lowenstein, J. M. (1988) *Biochem. J.* **253**, 117–121.
- Yamazaki, Y., Collinson, A. R., Truong, V. L. and Lowenstein, J. M. (1989) in *Purine and Pyrimidine Metabolism in Man, VI, Part B: Basic Research and Experimental Biology* (Mikanagi, K., Nishioka, K. and Kelley, W. N., eds.), pp. 107–111, Plenum Press, New York, London.
- Yamazaki, Y., Truong, V. L. and Lowenstein, J. M. (1991) *Biochemistry* **30**, 1503–1509.
- Skladanowski, A. C. and Newby, A. C. (1990) *Biochem. J.* **268**, 117–122.
- Darvish, A. and Metting, P. J. (1993) *Am. J. Physiol.* **264**, H1528–H1534.
- Burgess, F. W., El Kouni, M. H. and Parks, R. E. Jr., (1985) *Biochem. Pharmacol.* **34**, 3061–3070.
- Edwards, N. L., Recker, D., Manfredi, J., Rembecki, R. and Fox, I. H. (1982) *Am. J. Physiol.* **243**, C270–C277.
- Höglund, L. and Reichard, P. (1990) *J. Biol. Chem.* **265**, 6589–6595.
- Itoh, R., Usami, C., Nishino, T. and Tsushima, K. (1978) *Biochim. Biophys. Acta* **526**, 154–162.

- 16 Itoh, R. and Oka, J. (1985) *Comp. Biochem. Physiol.* **81B**, 159–163
- 17 Itoh, R. and Yamada, K. (1990) *Int. J. Biochem.* **22**, 231–238
- 18 Madrid-Marina, V. and Fox, I. H. (1986) *J. Biol. Chem.* **261**, 444–452
- 19 Madrid-Marina, V., Lestan, B., Nowak, P. J., Fox, I. H. and Spychala, J. (1993) *Leukaemia Res.* **17**, 231–240
- 20 Montero, J. M. and Fes, J. B. (1982) *J. Neurochem.* **39**, 982–989
- 21 Spychala, J., Madrid-Marina, V. and Fox, I. H. (1988) *J. Biol. Chem.* **263**, 18759–18765
- 22 Van den Berghe, G., van Pottelsberghe, Ch. and Hers, H.-G. (1977) *Biochem. J.* **162**, 611–616
- 23 Zekri, M., Harb, J., Bernard, S. and Meflah, K. (1988) *Eur. J. Biochem.* **172**, 93–99
- 24 Nass, N., Colling, Ch., Cramer, M., Genieser, H.-G., Butt, E., Winkler, E., Jaenicke, L. and Jastorff, B. (1992) *Biochem. J.* **285**, 129–136
- 25 Jastorff, B., Hoppe, J., Mato, J. M. and Konijn, T. M. (1979) in *Molecular Mechanisms of Biological Recognition* (Balaban, M., ed.), pp. 107–117, Elsevier/North Holland Biomedical Press, Amsterdam
- 26 Jastorff, B., Maronde, E., van Bemmelen, M. X. P., Zorn, M. and Störmann, R. (1991) in *Molecular Aspects of Chemotherapy* (Shugar, D., Rode, W. and Borowski, E., eds.), pp. 73–104, Polish Scientific Publishers/Springer Verlag, Warsaw
- 27 Genieser, H.-G., Butt, E., Bottin-Weber, U., Dostmann, W. and Jastorff, B. (1989) *Synthesis* **1**, 53–54
- 28 Worku, Y. and Newby, A. C. (1982) *Biochem. J.* **205**, 503–510
- 29 Smolenski, R. T., Lachno, D. R., Ledingham, S. J. M. and Yacoub, M. H. (1990) *J. Chromatogr.* **527**, 414–420
- 30 Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- 31 Itaya, K. and Ui, M. (1966) *Clin. Chim. Acta* **14**, 361–366
- 32 Dewar, M. J. S. and Thiel, W. (1977) *J. Am. Chem. Soc.* **99**, 4899–4906
- 33 Eckstein, F. (1985) *Annu. Rev. Biochem.* **54**, 367–402
- 34 Murray, A. W. and Atkinson, M. R. (1968) *Biochemistry* **7**, 4023–4029
- 35 Stolarski, R., Hagberg, C.-E. and Shugar, D. (1984) *Eur. J. Biochem.* **138**, 187–192
- 36 Orozco, M., Canela, E. I. and Franco, R. (1990) *Eur. J. Biochem.* **188**, 155–163
- 37 Jastorff, B., Hoppe, J. and Morr, M. (1979) *Eur. J. Biochem.* **101**, 555–561
- 38 Moréra, S., Lascu, J., Dumas, Ch., LeBras, G., Briozzo, P., Veron, M. and Janin, J. (1994) *Biochemistry* **33**, 459–467
- 39 Su, Y., Dostmann, W. R. G., Herberg, F. W., Durick, R., Xuong, N.-h., Ten Eyck, L., Taylor, S. S. and Varghese, K. J. (1995) *Science* **269**, 807–813
- 40 Newby, A. C. (1988) *Biochem. J.* **253**, 123–130
- 41 Bünger, R. and Soboll, S. (1986) *Eur. J. Biochem.* **159**, 203–213
- 42 Newby, A. C. (1984) *Trends Biochem. Sci.* **9**, 42–44
- 43 Skladanowski, A. C. (1988) in *Myocardial Energy Metabolism* (De Jong, J. W., ed.), pp. 53–65, Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster