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5′-Nucleotidase I from Rabbit Heart†

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ABSTRACT: 5′-Nucleotidase I (N-I) from rabbit heart was purified to homogeneity. After ammonium sulfate precipitation, the purification involved chromatography on phosphocellulose, DEAE-Sepharose, AMP–agarose, and ADP–agarose. The pure enzyme has a specific activity of 318 μmol (mg of protein)$^{-1}$ min^{-1}. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate yields a subunit molecular weight of 40 000. N-I is activated by ADP but not by ATP, in contrast to the 5′-nucleotidase (N-II) purified by Itoh et al. (1986), which is activated by ATP and, less well, by ADP. N-I displays sigmoidal saturation kinetics in the absence of ADP and hyperbolic kinetics in the presence of ADP. Partially purified N-I was previously shown to prefer AMP over IMP as substrate (Truong et al., 1988); this has been confirmed for pure N-I. Comparison of AMP and ADP concentrations reported to occur in heart with the kinetic behavior of N-I implicates N-I as the enzyme responsible for producing adenosine under conditions leading to a rise in ADP and AMP, such as hypoxia or increased workload. N-I is not activated by the ADP analogue adenosine 5′-methylenediphosphonate (AOPCP) and is only weakly inhibited by relatively high concentrations of AOPCP, in contrast to 5′-nucleotidase from plasma membrane, which is powerfully inhibited by this analogue. N-I shows an absolute dependence on Mg^{2+} ions. Mn^{2+} and Co^{2+} ions can replace Mg^{2+} ions as activator; Ni^{2+} and Fe^{2+} are much less effective, while Ca^{2+}, Ba^{2+}, Zn^{2+}, and Cu^{2+} fail to activate the enzyme.

Adenosine plays an important role in regulating coronary blood flow (Berne, 1980). It is released from heart cells during exercise, hypoxia, and ischemia and acts as a vasodilator. Adenosine is formed by the 5′-nucleotidase reaction and is destroyed principally by the adenosine deaminase reaction. In rat heart, the most active 5′-nucleotidase is the plasma membrane enzyme. The regulatory properties of this enzyme, such as its powerful inhibition by ADP (Naito & Lowenstein, 1981, 1985), as well as its outward-facing orientation, make it unlikely that it is the enzyme responsible for adenosine production under conditions when heart muscle increases its blood supply by using adenosine as a signal for coronary vasodilation. Moreover, studies of adenosine formation by heart cells in culture, in the presence and absence of inhibitors of adenosine transport, indicate that adenosine is formed intracellularly under these conditions (Meghji et al., 1985, 1988a,b; Altschuld et al., 1987). A cytosolic 5′-nucleotidase was isolated by Itoh and co-workers and thought to be responsible for generating intracellular adenosine (Itoh & Oka, 1985; Itoh et al., 1986). However, this enzyme has a much higher affinity for IMP than for AMP and is activated strongly by ATP and less strongly by ADP. In the heart, $[ADP]_{free}$ rises substantially in response to increased workload, hypoxia, and ischemia, while [ATP] drops. The enzyme responsible for producing adenosine under

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these conditions might therefore be expected to be activated by ADP but not by ATP. The properties of the enzyme isolated by Itoh et al. (1986) make it unlikely that it is the major catalyst for generating adenosine; instead, it appears to be tailored to dephosphorylate IMP.

Rat heart contains two different, soluble 5′-nucleotidases, which we have termed N-I and N-II on the basis of their order of elution upon phosphocellulose chromatography. N-I prefers AMP over IMP as substrate, while N-II prefers IMP over AMP (Truong et al., 1988). An enzyme similar to N-I has been found in pigeon heart (Skladanowski & Newby, 1990). After N-I had been separated from N-II by phosphocellulose chromatography, N-I appeared to be activated equally well by ATP and ADP (Collinson et al., 1987); however, the lack of specificity toward ATP and ADP was an artifact, because the peak of N-I activity obtained by phosphocellulose chromatography was contaminated by myokinase. We now report the purification to homogeneity of N-I from rabbit heart. The pure enzyme is activated by ADP, but not by ATP. Comparison of the affinity of N-I for ADP with physiological levels of ADP under different conditions suggests that ADP is a key regulator of N-I in vivo. Other properties of N-I are described and compared with other soluble 5′-nucleotidases.

Materials and Methods

Frozen rabbit hearts were obtained from Pel-Freez Biochemicals. Cellulose phosphate P11 was from Whatman. DEAE-Sepharose CL-6B was from Pharmacia. AMP–agarose, ADP–agarose [agarose-NH-$(CH_2)_6$-N^6-adenosine 5′-phosphate or diphosphate, respectively], bovine serum albumin, ovalbumin, carbonic anhydrase from bovine erythrocytes, cytochrome *c* from horse heart, and phosphorylase from rabbit heart were purchased from Sigma, as were all protease inhibitors.

Purification of N-I. All procedures were carried out at 0–5 °C unless otherwise indicated. Buffer A consisted of the following mixture: 40 mM sodium *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate (NaHEPES)[1] buffer, pH 7.0, 25% glycerol (v/v), 1 mM DTT, 1 mM EDTA, and 0.2 mM PMSF. Buffer A+P contained in addition the following protease inhibitors: 0.27 mg/L antipain, 1.8 mg/L pepstatin, 0.90 mg/L leupeptin, and 0.36 mg/L L-1-(tosylamino)-2-phenylethyl chloromethyl ketone (TPCK).

Step 1: Preparation of Heart Extract and Ammonium Sulfate Precipitation. Frozen rabbit hearts (typically 2 kg) were pulverized in a stainless-steel percussion mortar cooled with liquid nitrogen. The power was suspended in 4 mL of buffer A+P per gram of tissue and homogenized in a Polytron (Brinkmann) at maximum speed using three bursts of 45-s duration, with a 2-min pause between each burst. The homogenate was centrifuged at 14000*g* for 45 min. The supernatant was filtered through gauze and centrifuged at 150000*g* for 60 min. Solid ammonium sulfate (351 g/L) was added to the supernatant with stirring. The mixture was allowed to stand for 4 h, and the suspension was centrifuged at 14000*g* for 60 min. The resulting precipitate was suspended in a minimum volume of buffer A and dialyzed against buffer A overnight with two changes of buffer.

Step 2: Phosphocellulose Chromatography. The dialyzed ammonium sulfate fraction from step 1 was applied to a phosphocellulose column (2.6 × 36 cm) equilibrated with buffer A. The column was washed with 400 mL of buffer A and eluted with a linear gradient of NaCl from 0 to 800 mM over 1200 mL in buffer A. Fractions containing N-I activity were pooled. Solid ammonium sulfate (472 g/L) was added to the pooled fractions, and the suspension was centrifuged at 14000*g* for 60 min. The resulting precipitate was suspended in a minimum volume of buffer A and dialyzed against buffer A overnight, with two changes of buffer.

Step 3: DEAE-Sepharose CL-6B Chromatography. The dialyzed sample from step 2 was applied to a column of DEAE-Sepharose CL-6B (1.5 × 19 cm) equilibrated with buffer A. The column was washed with 300 mL of buffer A and eluted with a linear gradient of NaCl from 0 to 0.2 M over 350 mL of buffer A.

Step 4: AMP–Agarose Chromatography. The fractions containing N-I from step 3 were combined and precipitated with ammonium sulfate (472 g/L). The precipitate was recovered by centrifugation, suspended in buffer A, and dialyzed against buffer A overnight. The protein fraction so obtained was applied to an AMP–agarose column (1 × 6.5 cm) equilibrated with buffer A. The column was washed with 50 mL of buffer A and eluted with a linear gradient of AMP from 0 to 20 mM in 100 mL of buffer A.

Step 5: ADP–Agarose Chromatography. The fractions containing N-I activity from step 4 were stored in 10 mM AMP in buffer A. These fractions were diluted 4-fold with buffer A and then applied to an ADP–agarose column (1 × 6.5 cm) equilibrated with buffer A. The column was then washed with 40 mL of buffer A containing 10 mM $MgCl_2$ and eluted with a linear gradient going simultaneously from 0 to 15 mM AMP and from 0 to 600 mM NaCl over 80 mL of buffer A.

Assay of 5′-Nucleotidase. The reaction mixture contained 50 mM NaMOPS buffer, pH 6.9, 5 mM AMP, 3 mM $MgCl_2$, and 1 mM ADP in a final volume of 0.5 mL. The reaction was started by adding enzyme and was run at 37 °C. The reaction was stopped by adding 2 mL of ice-cold, 10% trichloroacetic acid. Precipitated protein was removed by centrifugation, and 2 mL of the supernatant fluid was used for orthophosphate determination by the method of Sanui (1974). One unit is the amount of enzyme that hydrolyzes 1 μmol of AMP per minute. Unless otherwise indicated, the assays were run under conditions where the reaction rate was linear with time and enzyme concentration.

Other Assays. The following enzyme assays were also used: N-II, assay 1 of Itoh and Oka (1985), except than 50 mM MOPS-HCl buffer, pH 6.9, was used in place of imidazole hydrochloride buffer, pH 6.5, and 5 mM IMP was used in place of 3 mM; myokinase, Whitesides et al. (1976); nonspecific phosphatase, Truong et al. (1988), except that 50 mM MOPS-HCl buffer, pH 6.9, was used in place of acetate buffer. Protein was assayed according to Bradford (1976), using bovine serum albumin as standard.

Sodium Dodecyl Sulfate Gel Electrophoresis. Electrophoresis in the presence of sodium dodecyl sulfate was performed using 10% polyacrylamide gel according to the method of Laemmli and Favre (1973). Molecular weight standards employed ($\times 10^{-3}$) were phosphorylase (97.4), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (29), and cytochrome *c* (12.4).

Results

Purification of N-I. Results of a typical purification are shown in Table I. The pure enzyme had a specific activity of 318 μmol mg^{-1} min^{-1} with AMP as substrate. This is the

[1] Abbreviations: AOPCP, α,β-methylenediphosphonate analogue of ADP; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

Table I: Purification of N-I from Rabbit Heart[a]

step	protein (mg)	activity (units)	specific activity (units/mg)	% yield between steps	% yield overall
(1) ammonium sulfate	8400 (8400)				
(2) phosphocellulose	1194 (844)	597 (573)	0.50 (0.68)	100	96
(3) DEAE-Sepharose CL-6B	111 (45)	505 (401)	4.5 (8.9)	88	85
(4) AMP-agarose	8.6 (0.57)	375 (30.1)	43.6 (52.8)	94	63
(5) ADP-agarose	0.073	23.2	318	77	48[b]

[a] The purification started with 2.29 kg of frozen rabbit hearts. 5'-Nucleotidase was assayed as described under Materials and Methods using protein concentrations for which activity was linear with respect to time of incubation. One unit of activity corresponds to the liberation of 1 μmol of P_i per minute. Total activity and protein recovered are shown for each step. The amounts actually taken for the next step are shown in parentheses. Activity and yield are not given for step 1, because nonspecific phosphatases were present in the ammonium sulfate precipitate. [b] Only 30.1 units or 8% of the activity recovered in step 4 (fractions 14–16 from the ADP-agarose column) were applied to the column in step 5. Step 5 was repeated a number of times with other fractions from step 4 with similar results. The overall yield was calculated assuming that all of the activity recovered in step 4 was used for step 5.

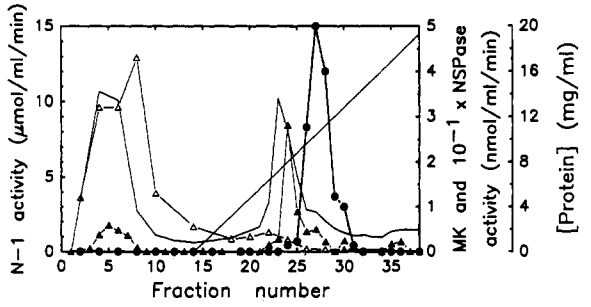

FIGURE 1: DEAE-Sepharose CL-6B column chromatography of N-I. The peak of N-I activity obtained from the phosphocellulose chromatography step was concentrated as described under Materials and Methods and applied to the DEAE column (1.5 × 19 cm). The column was washed with 300 mL of buffer A and then eluted with a linear gradient of NaCl from 0 to 200 mM in buffer A over 350 mL. Fractions 1–5 contained 20 mL each; fractions 16–40 contained 14.6 mL each. The following activities were measured: (●) N-I; (▲) nonspecific phosphatase (NSPase); (△) myokinase (MK). The curve without symbols shows the protein concentration. N-II activity was also measured in the fractions, but none was found. The straight line shows the NaCl gradient.

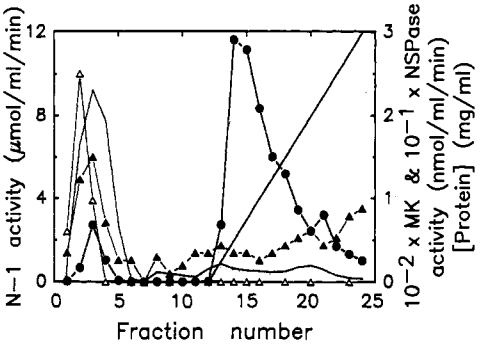

FIGURE 2: AMP-agarose column chromatography of N-I obtained from the DEAE-Sepharose column. The peak of N-I activity shown in Figure 1 was concentrated and applied to the AMP-agarose column (1 × 6.5 cm). The column was washed with 50 mL of buffer A and then eluted with a linear gradient of AMP from 0 to 20 mM in buffer A over 80 mL. Fractions contained 6.7 mL. The symbols are as for Figure 1. The straight line shows the AMP gradient which had reached 20 mM at fraction 24.

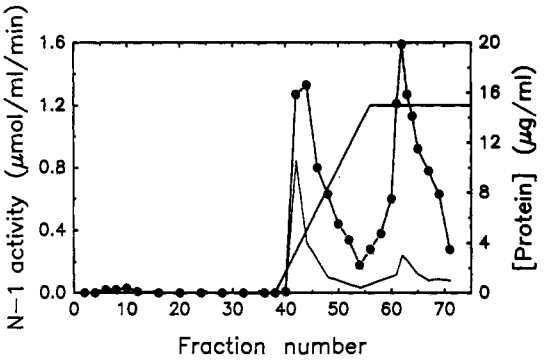

FIGURE 3: ADP-agarose column chromatography of N-I obtained from the AMP-agarose column. The peak of N-I activity shown in Figure 2 was concentrated by precipitation with 75% ammonium sulfate, dissolved in a minimum of buffer A, and dialyzed against buffer A overnight. The resulting solution was applied to an ADP-agarose column (1 × 6.5 cm). The column was washed with buffer A and then with 40 mL buffer A containing 10 mM $MgCl_2$. It was then eluted with a gradient going simulataneously from 0 to 15 mM AMP and from 0 to 600 mM NaCl over 80 mL. Elution with 15 mM AMP and 600 mM NaCl in buffer A was then continued as indicated. Fractions contained 4.4 mL. The symbols are as for Figure 1. The straight line shows the start and end of the AMP–NaCl gradient, followed by constant concentrations of both.

highest value reported for a soluble 5'-nucleotidase from animal tissues. Eight similar purifications have been carried out with similar results.

The activity of N-I in cytosol and in the first ammonium sulfate fraction is difficult to measure, because of the presence of relatively high activities of nonspecific phosphatases. Accordingly, activities in Table I are quoted only starting with step 2. The separation of N-I from N-II and nonspecific phosphatases by chromatography on phosphocellulose was shown previously (Truong et al., 1988). It was found subsequently that the N-I peak is sandwiched between two overlapping peaks of myokinase activity. The presence of myokinase in the N-I peak explains the apparent activation of N-I by ATP which was observed previously (Truong et al., 1988). Myokinase is largely removed during step 2, which consists of chromatography on DEAE-cellulose (Figure 1). The remainder is removed completely during step 3, which consists of chromatography on AMP-agarose (Figure 2). The final purification occurs during step 4 which involves chromatography on ADP-agarose. N-I can be eluted from ADP-agarose columns either with a gradient of NaCl and ADP or with a gradient of NaCl and AMP. Figure 3 shows elution of the enzyme with an NaCl–AMP gradient. Two peaks of activity are observed. The second peak yields a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with an apparent molecular weight of 40000 (Figure 4). The first peak shows multiple bands on gel electrophoresis, among which a band of apparent M_r 40000 predominates (not shown in Figure 4).

N-I is unstable in crude extracts unless 25% glycerol is present. After chromatography on AMP-agarose, N-I is stable when stored unfrozen at 0 °C for 2 months, provided that 25% glycerol and at least 6 mM AMP are present. After chromatography on ADP-agarose, N-I is unstable even in 25% glycerol and 6 mM AMP, presumably because the enzyme

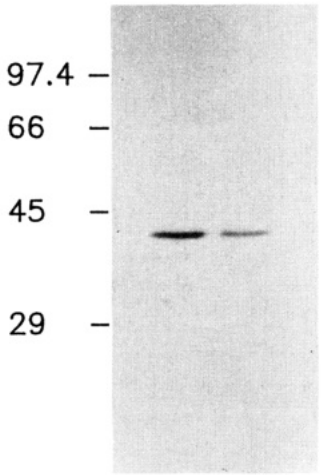

FIGURE 4: Polyacrylamide gel electrophoresis of N-I in the presence of sodium dodecyl sulfate. The peak fractions from the second peak in Figure 3 were precipitated with trichloroacetic acid. Electrophoresis on the dissolved precipitates was performed as described under Materials and Methods, followed by silver staining. Subunit molecular weights ($\times 10^{-3}$) of marker proteins: phosphorylase (97.4), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (29).

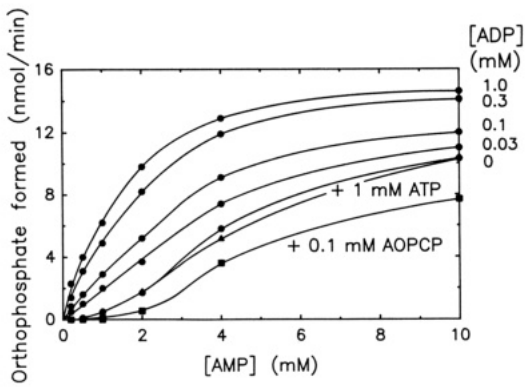

FIGURE 5: Effect of ADP, ATP, or AOPCP on N-I activity as a function of AMP concentration. Reaction mixtures contained 50 mM NaMOPS buffer, pH 6.9, 3 mM $MgCl_2$, 0.09 unit of enzyme, and AMP as indicated in a total volume of 0.5 mL. The reactions were run at 37 °C in the presence of (●) ADP as indicated, (▲) 1 mM ATP, or (■) 0.1 mM AOPCP.

is highly diluted. It can be stabilized by adding 0.1% bovine serum albumin, but even then loses activity slowly.

Effect of ADP, ATP, and AOPCP. N-I exhibits a sigmoidal AMP saturation curve in the absence of ADP and a hyperbolic curve in the presence of sufficiently high concentrations of ADP (Figure 5). In other words, ADP increases the apparent affinity of the enzyme for AMP. ATP has no effect on the activity of N-I. However, some batches of ATP show slight activation at millimolar concentrations, because they are contaminated with small amounts of ADP. Note that ADP and, to a lesser degree, ATP are powerful inhibitors of the plasma membrane enzyme. AOPCP, which is the most powerful known inhibitor of the plasma membrane enzyme, with a K_I of 6 nM (Burger & Lowenstein, 1975; Naito & Lowenstein, 1985), has little or no effect on N-I even at 0.1 mM. In the presence of 0.5 mM AMP, half-maximal activation of N-I occurs at about 10 μM ADP (Figure 6). At lower AMP concentrations, the apparent affinity of the enzyme for ADP is decreased, that is to say, half-maximal activation occurs at higher concentrations of ADP (not shown in Figure 6). Under the conditions of the experiment shown in Figure 6, about two-thirds of the ADP is chelated to Mg^{2+}, and the remainder is unchelated. These calculations were made by using the

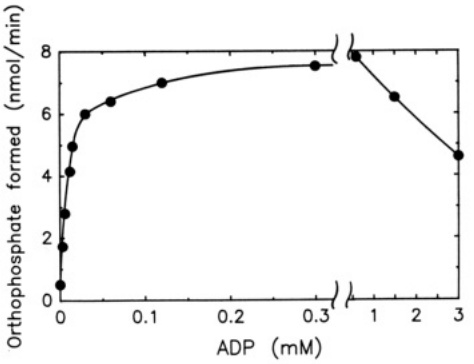

FIGURE 6: N-I activity as a function of ADP concentration. Reaction mixtures contained 50 mM NaMOPS buffer, pH 6.9, 0.5 mM AMP, 1 mM free Mg^{2+} (added in the form of $MgCl_2$), 0.09 unit of enzyme, and ADP as indicated in a total volume of 0.5 mL. The reaction was run at 37 °C. The amount of $MgCl_2$ added to each reaction mixture to yield 1 mM free Mg^{2+} was calculated by using the stability constants listed by Goodman and Lowenstein (1977).

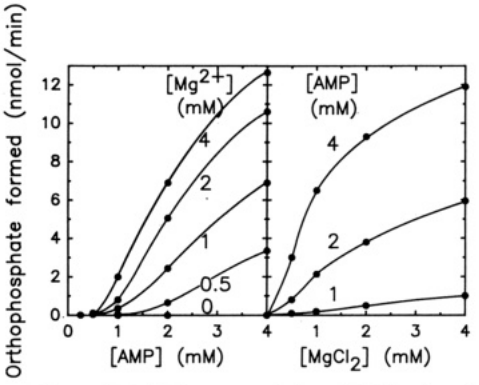

FIGURE 7: Effect of Mg^{2+} ions on activity of N-I in the absence of ADP. Reaction mixtures contained 50 mM NaMOPS buffer, pH 6.9, AMP and Mg^{2+} as indicated, and 0.09 unit of enzyme, in a total volume of 0.5 mL. The reaction was run at 37 °C.

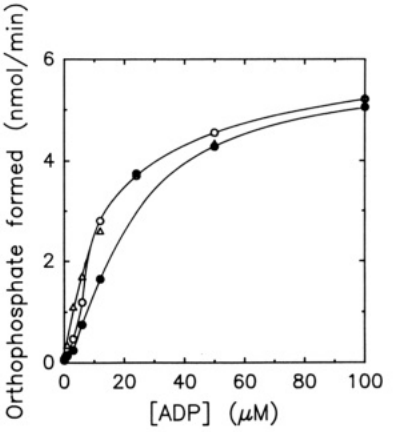

FIGURE 8: Effect of Mg^{2+} ions on activity of N-I in the presence of ADP. Reaction mixtures contained 50 mM NaMOPS buffer, pH 6.9, 0.5 mM AMP, and 0.09 unit of enzyme, in a total volume of 0.5 mL. The reaction mixture also contained free Mg^{2+} at the following concentrations: (●) 1 mM; (○) 2 mM; (△) 4 mM. The reaction was run at 37 °C. Free [Mg^{2+}] was calculated as mentioned in the legend to Figure 7.

equations and stability constants described previously (Goodman & Lowenstein, 1977). N-I from the AMP–agarose and ADP–agarose fractions (Table I) shows the same kinetic behavior. Pure N-I is unstable in high dilutions, such as are used in kinetic experiments; for this reason, care was taken to establish that the measured rates were linear with time for all experimental points.

Table II: Comparison of Properties of Pure 5′-Nucleotidases from Various Sources[a]

source	subunit M_r ($\times 10^{-3}$)	activators	K_{app} (mM)	inhibitors	K_I (μM)	references
rat heart plasma membrane	72	Mg^{2+} at pH >7.9	0.5–1.6	ADP	0.082	Naito & Lowenstein (1981, 1985)
				ATP	1.5	
				AOPCP	0.006	
rat liver plasma membrane	73[b]	nt		nt		Misumi et al. (1990)
cow liver						
plasma membrane	70	Mg^{2+}	~2	nt		Harb et al. (1983)
	70	nt		nt		Zekri et al. (1988)
cytosol	65, 57	ATP ≈ ADP	ng			
		Mg^{2+}	ng			
chicken gizzard						
smooth muscle	79			ADP	2.9	Dieckhoff et al. (1985)
membranes				ATP	15	
				AOPCP	0.019	
human placenta						
cytosol (low K_{app})	76	Mg^{2+}	~10	ADP	15	Madrid-Marina & Fox (1986)
				ATP	100	
cytosol (high K_{app})	53	ATP	0.7–2.2[c]			Spychala et al. (1988)
chicken liver cytosol (N-II)[d]	70	ATP				Oka et al. (1988)
		ADP				
		Mg^{2+}				
rat heart cytosol (N-I)	40	ADP	0.025			this paper
		Mg^{2+}	~2.5			

[a] Preparations are listed for which specific activity and gel electrophoresis indicate a high degree of purity and which, in most cases, were tested for the effects of ADP and ATP on activity. ng, not given; nt, not tested. [b] The molecular weight obtained from gel electrophoresis in SDS was 73 000; the molecular weight calculated from two cDNA clones was 63 965. The source of the enzyme was stated to be microsomes, but it appears to be identical with the plasma membrane enzyme. [c] Depends on IMP concentration. [d] A similar enzyme occurs in chicken heart cytosol (Itoh & Oka, 1985) and rat liver cytosol (Itoh, 1981a,b, 1982).

Effect of Mg^{2+} Ions. N-I shows an absolute dependence on Mg^{2+} ions both in the presence and in the absence of ADP (Figures 7 and 8). The Mg^{2+} concentrations used in these experiments were chosen to cover the extremes reported for the free ion in various organs. Figure 7 shows that with <0.5 mM AMP and <3 mM Mg^{2+}, the enzyme is inactive in the absence of ADP, in other words, that it is inactive under physiological conditions, unless sufficient ADP is also present. Figure 8 shows that at ADP concentrations of <25 μM, N-I activity is somewhat sensitive to changes in [Mg^{2+}]; however, above 25 μM ADP, N-I becomes less sensitive or insensitive to changes of [Mg^{2+}] in the physiological range.

Tests of other bivalent metal ions showed that Mn^{2+} and Co^{2+} can take the place of Mg^{2+}, but are somewhat less effective as activators of N-I. Ni^{2+} and Fe^{2+} are weak activators, while Ca^{2+}, Ba^{2+}, Zn^{2+}, and Cu^{2+} fail to activate N-I.

Effect of pH. N-I activated by ADP shows a pH optimum of 6.5–6.7; its activity is diminished by 8, 18, 29, and 36% at pH 6.9, 7.1, 7.3, and 7.5, respectively.

Specificity Comparison of N-I and N-II. The different specificities of N-I and N-II toward AMP and IMP were originally described for impure preparations of N-I which were contaminated with myokinase (Truong et al., 1988). The enzyme showed preference for AMP over IMP, whereas the converse was true for N-II. These experiments have been repeated with pure N-I, free of myokinase, in the presence and absence of ADP as activator, with similar results.

Discussion

N-I from heart cytosol is the first 5′-nucleotidase to be purified which is activated by ADP but unaffected by ATP (Figure 5). Pure N-I has a specific activity of 318 μmol mg^{-1} min^{-1} with AMP as substrate. Pure N-II was reported to have a specific activity of 28.5 μmol mg^{-1} min^{-1} with IMP as substrate (Itoh et al., 1986). In confirmation with our previous finding (Truong et al., 1988), N-I has a higher affinity for AMP than IMP (not shown). This is in contrast to N-II from heart cytosol which has a much higher affinity for IMP than AMP. In contrast, 5′-nucleotidase from plasma membrane of rat heart is inhibited strongly by ADP and ATP (Naito & Lowenstein, 1985). Thus, heart possesses at least three different types of 5′-nucleotidase.

A partially purified, soluble 5′-nucleotidase from pigeon heart was reported to show a preference for AMP over IMP (Gibson & Drummond, 1972). ATP inhibited this enzyme at high conformations and activated at low concentrations of AMP. At first sight, such kinetic behavior would appear to rule out that this pigeon heart enzyme is similar to N-I from rabbit heart; however, the relatively crude preparation used by Gibson and Drummond (1972) was undoubtedly contaminated with myokinase and nonspecific phosphatases. These interfering enzymes would have given an incorrect picture of the kinetic behavior of the 5′-nucleotidase studied by these authors. Newby (1988) and Skladanowski and Newby (1990) have reported a partially purified, soluble 5′-nucleotidase from pigeon heart that prefers AMP over IMP and is activated by ATP and ADP. Their crude pigeon heart preparation showed a behavior similar to that of Gibson and Drummond (1972), while their partially purified enzyme showed less activation by ATP. This suggests to us that the partially purified pigeon enzyme was less contaminated by adenylate kinase.

Table II provides a comparison of various pure 5′-nucleotidases described in the literature. It is apparent that N-I differs from the pure enzymes obtained previously. The amino acid sequence of the plasma membrane enzyme has been deduced from its DNA sequence (Misumi et al., 1990). The first proteolytic peptide of N-I to be sequenced shows no homology with the plasma membrane enzyme (Morgan and Lowenstein, unpublished observations).

Human erythrocytes contain soluble 5′-nucleotidases which can be separated into three peaks on a DEAE-Trisacryl column. The enzyme in peak 1 shows a preference for deoxyIMP over IMP and AMP. The enzyme in peak 2 shows preference for IMP over AMP and is activated by ATP and 2,3-diphosphoglycerate. The enzyme in peak 3, which runs between peaks 1 and 2, shows an absolute dependence on 2,3-di-

phosphoglycerate as activator; it was not investigated further (Bontemps et al., 1988). A soluble 5′-nucleotidase from placenta is inhibited by ADP and ATP (Madrid-Marina & Fox, 1986), but much less strongly that the ectoenzyme from heart (Naito & Lowenstein, 1985). Soluble 5′-nucleotidases from bull seminal plasma can also be resolved into three peaks of activity; however, these appear to be artifacts of aggregation, because they are converted to one form by detergents (Fini et al., 1983). A soluble nucleotidase has been purified to homogeneity from human placenta (Höglund & Reichard, 1990); it shows much higher activity with deoxyribonucleoside 5′-phosphates than with ribonucleoside 5′-phosphates, but is not a true 5′-nucleotidase, because it has high activity with ribonucleoside 2′- and 3′-phosphates.

In muscle, cytosolic $[ADP]_{free}$ cannot be determined after deproteinization. Most of the ADP in heart is bound tightly to actin and is released by deproteinization. $[ADP]_{free}$ can, however, be calculated from ATP, creatine, and creatine phosphate contents, assuming that the creatine kinase and adenylate kinase reactions are in equilibrium (Goodman & Lowenstein, 1977; Bünger & Soboll, 1986). ATP and creatine phosphate concentrations were measured in perfused guinea pig heart under various conditions using ^{31}P NMR. Concentrations of free ADP were calculated from the data so obtained, assuming that the total creatine content remained constant and that the creatine kinase reaction was at or near equilibrium. Control levels of $[ADP]_{free}$ were 4–10 μM. $[ADP]_{free}$ rose to a maximum of 30 μM upon infusion of norepinephrine. Adenosine release was 11 pmol g^{-1} min^{-1} before and 250 pmol g^{-1} min^{-1} during epinephrine infusion. The release of adenosine followed the rise in [ADP] closely (He et al., 1987). Experiments with perfused guinea pig hearts showed that $[ADP]_{free}$ was 35 and 58 μM for organs perfused with 16 mM glucose or 10 mM pyruvate, respectively (Zweier & Jacobus, 1987). Adenosine output was not measured in these experiments. Experiments using ^{31}P NMR with dog heart in vivo showed no significant change in the calculated $[ADP]_{free}$ between control and high rates of oxygen uptake, both being high, namely, 50–60 μM (Katz et al., 1989). Concentrations of ADP_{free} in guinea pig heart were calculated from metabolite contents determined enzymatically after nonaqueous homogenization and fractionation into organelles and cytosol. Under these conditions, ADP_{bound} remains bound to actin. $[ADP]_{free}$ was 23 and 48 μM during control and high rates of oxygen uptake, respectively (Bünger & Soboll, 1986). These measurements indicate that in the intact heart, ADP_{free} undergoes changes in concentration consistent with being a regulator of N-I activity.

The concentration of Mg^{2+}_{free} in beating guinea pig and dog heart was 2.5 mM as determined by ^{31}P NMR; it was not changed by actions which change coronary flow (Wu et al., 1981; Katz et al., 1989). In contrast, Headrick and Willis (1989), who also used ^{31}P NMR, found a rise in $[Mg^{2+}]_{free}$ from 0.76 to 2.4 mM in response to epinephrine and from 0.75 to 2.7 mM in response to hypoxia. Figure 8 shows that such a rise would result in a small increase in N-I activity when [ADP] is <25 μM.

Registry No. ADP, 58-64-0; AMP, 61-19-8; Mg, 7439-95-4; nucleotidase, 9027-73-0.

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Isolation and Characterization of Somatolactin, a New Protein Related to Growth Hormone and Prolactin from Atlantic Cod (*Gadus morhua*) Pituitary Glands†

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ABSTRACT: The characterization of cod somatolactin (SL), a new pituitary protein belonging to the growth hormone/prolactin family, is described. Cod SL has a molecular weight of 26 kDa and consists of 209 amino acids, of which eight are Cys. The protein has three disulfide bonds between residues Cys^{5}–Cys^{15}, Cys^{65}–Cys^{181}, and Cys^{198}–Cys^{206}. The Cys residues at positions 42 and 180 are not involved in disulfide bonding. The positions of these disulfide bonds are homologous to those found in prolactin and growth hormone. Cod SL has two possible N-glycosylation sites, but only one appears to have carbohydrate units attached. Chemical analysis showed the following sugars to be present: galactose, mannose, *N*-acetylneuramic acid, and glucosamine. A smaller variant (23 kDa) of SL has been isolated, which is believed to be deglycosylated. Sequence comparison revealed cod SL to be similarly related to both GH and PRL, but slightly higher identity was observed to the tetrapod hormones (27–33%) than to the teleost hormones (21–27%).

The pituitary hormones growth hormone (GH)[1] and prolactin (PRL) and placental lactogen (PL) of placental origin are structurally related and grouped together in the GH/PRL family. This family has recently been extended due to the discovery of placental proteins such as mouse proliferin (Linzer & Nathans, 1984), bovine PRL related cDNA I (Schuler & Hurley, 1987), and rat prolactin like protein A (Deb et al., 1989). These placental proteins, with the exception of human PL, have been found to be structurally more similar to PRL (30–40%) than to GH (15–21%), indicating that they may have evolved from a PRL gene (Duckworth et al., 1988). Human PL on the other hand, shows 85% identity with human GH (Miller & Eberhardt, 1983). A recent study has also isolated GH-related (rather than PRL-related) proteins from rat placenta (Ogilvie et al., 1990).

Recently, we discovered somatolactin (SL), a novel protein in pituitaries from Atlantic cod and flounder (Ono et al., 1990; Rand-Weaver et al., 1991). Analysis of the flounder cDNA sequence revealed it to be distant and similarly related to GH and PRL and thus the first new hypophyseal member of the GH/PRL family (Ono et al., 1990). By use of an antiserum raised against cod SL, the protein was shown to be produced in the pars intermedia of all teleosts tested (Rand-Weaver et al., 1991). Specifically, SL immunostaining was observed in the PAS-positive cells of cod, flounder, molly, killifish, and catfish and the chromophobic cells of rainbow trout. The PAS-positive cells have been considered to produce a factor involved in such diverse functions as calcium regulation (Ball et al., 1982; Olivereau et al., 1985), acid–base metabolism (Wendelaar-Bonga et al., 1986), adaptation to background (Ball & Batten, 1981), and reproduction [quoted in Ball and Baker (1969)].

We report here a simplified isolation procedure of cod SL and its characterization and primary structure determination. The relationship of cod SL with other members of the GH/PRL superfamily is discussed.

Materials and Methods

Isolation. Pituitary glands were taken from cod (*Gadus morhua*) caught in the Norwegian Sea (Lofoten), Norway, during February. They were frozen immediately on dry ice and stored at −80 °C until used. The glands were extracted with 0.05 M ammonium acetate, pH 9.0, for 1 h at 4 °C and chromatographed on Sephadex G-75 (1.9 × 100 cm) equilibrated and eluted in extraction buffer. Final purification of SL was achieved on a TSK gel ODS-120T reverse-phase (rp) HPLC column (0.46 × 25 cm, particle size 5 μm). Elution was performed by using a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1 mL/min, and absorbance was monitored at 220 nm.

Electrophoresis. The molecular weight was estimated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) performed before and after reduction with

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[1] Abbreviations: GH, growth hormone; PRL, prolactin; PL, placental lactogen; SL, somatolactin; PAS-positive cells, periodic acid–Schiff positive cells; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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