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Temperature Effects on the Structural and Functional Properties of GPI-Anchored and Anchor-Less Bull Seminal Plasma Ecto-5′-Nucleotidase[1]

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The effects of temperature on the three-dimensional organization and on the secondary structure of GPI-anchored 5′-nucleotidase from bull seminal plasma and of its anchor-less form (solubilized ecto-5′-nucleotidase), obtained after GPI anchor removal by phosphatidylinositol-specific phospholipase C were investigated in parallel by circular dichroism and fluorescence spectroscopy. The structural features of the two enzymes were correlated to their functional properties in the temperature range of 25-90°C. The kinetic data indicated that the enzyme activities were temperature dependent, showing the maximal values at 60°C. The relevant Arrhenius plots were linear in the temperature range of 20-60°C and the activation energies were 44.4 and 51.8 kJ/mol for the solubilized and GPI-anchored 5′-nucleotidase, respectively. The time-course measurements of enzyme activity, in the temperature range of 25-55°C, revealed that the two enzymes were of different thermal stability, the solubilized ectoenzyme showing lower thermal deactivation constants and longer half lives. Fluorescence and near UV circular dichroism spectroscopy showed that temperature increases induced remarkable changes in the protein tertiary structure of the two enzymes, whereas far-UV circular dichroism analysis revealed only a small temperature effect on the protein secondary structure content.

Key words: circular dichroism, fluorescence spectroscopy, 5′-nucleotidase, seminal plasma, thermostability.

5′-Nucleotidase occurs in cells both as a glycosyl phosphatidylinositol membrane-anchored ectoenzyme and a cytosolic soluble protein devoid of the GPI moiety. In addition, a soluble 5′-nucleotidase isoform of extracellular location and derived from the GPI-anchored enzyme after removal of the GPI moiety by endogenous phospholipases has also been described (*1*). The distribution of 5′-nucleotidase activity in the olfactory bulb of adult rat has recently been described in detail by Schoen and Kreutzberg (*2*), who suggested that the enzyme has roles in synaptic plasticity, in cell recognition and in adhesion, as well as in purinergic neuromodulation.

Ecto-5′-nucleotidase has been found to be active in the intrarenal formation of adenosine *via* an extracellular pathway which also involves cAMP (*3*). Moreover, the GPI anchor has been shown to be non-essential for signal transduction in T cell activation through the differentiation antigen CD73 (*4*), and the enzyme has been described an accessory T-lymphocyte activation molecule (*5*). IMP/GMP- and AMP-preferring soluble cytosolic 5′-nucleotidases have been found in various mammalian sources, and they show K_m values in the millimolar range. The IMP-preferring enzyme has been termed "high-K_m" 5′-nucleotidase, and it is stimulated by ATP and ADP. The AMP-preferring enzyme has similar properties, except that it is activated only by ADP with a decrease of the K_m towards micromolar values (*1*). A 5′-nucleotidase highly specific for IMP has been purified from baker's yeast. This preference for IMP over other nucleoside monophosphates, together with deactivation of the enzyme under conditions of ATP depletion, suggested a role for the enzyme in the modulation of the intracellular IMP/inosine levels (*6*). An intriguing bifunctional role has recently been described for the cytosolic high-K_m 5′-nucleotidase from calf thymus. In the presence of physiological concentrations of AMP the enzyme appears to behave like a transferase, being able to transfer, *via* a phosphorylated intermediate, the phosphate group from various ribo- and deoxyribonucleoside monophosphates to available nucleoside acceptors (*7*).

Molecular characterization studies have shown that ecto-5′-nucleotidase consists of a homodimeric protein with two disulfide-linked subunits of 70-79 kDa, each containing one GPI moiety (*1* and references cited therein). However, $\alpha\alpha$-dimers as well as $\alpha\beta$-dimers ($\alpha = 70$ kDa, $\beta = 38$ kDa) have been described for 5′-nucleotidase from rat liver (*8*). The cleavage of the disulfide bridges by thiol reagents resulted in the complete loss of the AMPase activity of 5′-nucleotidase from bull seminal plasma (*9*). The real number of disulfide bridges has not been established as yet. In addi-

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Abbreviations: CD, circular dichroism; GPI, glycosyl phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.

tion, 5′-nucleotidase from chicken liver (*10*), rat heart (*11*), calf thymus (*12*), and yeast (*6*) could be extracted in a molecular form of about 220 kDa. Reducing SDS-PAGE resulted in a single protein band of 51-59 kDa, indicating that the native enzyme is constituted of four subunits. A heterodimer of about 130 kDa cytosolic 5′-nucleotidase has been purified from bovine liver (*13*).

cDNA analysis has provided the amino acid sequences for a number of 5′-nucleotidases. Homology searching for primary and secondary structures revealed striking similarities among 5′-nucleotidase from electric ray brain (*14*), rat liver (*15*), and human placenta (*16*). Furthermore, homologous regions were found by comparing the amino acid sequences with those of nucleotide-hydrolyzing enzymes such as rat liver 5′-nucleotidase, UDP-glucose hydrolase, and 2′,3′-cyclic phosphodiesterase (*16*). However, in spite of the extensive information gained in the last few years on the molecular, kinetic, and physiological properties of 5′-nucleotidase, many of its structural features still remain to be defined.

In this study, we examined the thermal behavior of GPI-anchored bull seminal plasma 5′-nucleotidase and of its soluble form prepared by PI-PLC treatment. In a previous FT-IR thermal analysis (*17*), we showed that temperatures in the range of 60-65°C induced the aggregation of GPI-anchored bull seminal plasma 5′-nucleotidase. However, it is important to point out that this technique requires highly concentrated protein solutions (3-5%, w/v) and consequently protein aggregation phenomena can easily take place. To overcome this problem, we investigated the temperature effects on the structural features of 5′-nucleotidase by using fluorescence spectroscopy and circular dichroism, techniques requiring much lower protein concentrations (0.002-0.005%, w/v). Moreover, in order to demonstrate whether or not the GPI anchor is involved in the functional and structural properties of 5′-nucleotidase, we compared the temperature effect on the GPI-anchored enzyme to that on the soluble enzyme obtained by removing the GPI anchor from the native ecto-5′-nucleotidase of bull seminal plasma. This work stemmed from an incidental observation that at temperatures as high as 57-65°C the GPI-enzyme showed maximum velocity of AMP hydrolysis with well-maintained catalytic efficiency.

MATERIALS AND METHODS

Materials—Bull semen was kindly provided by Dr. Franca Farabegoli (Societã Semen Italy, Centro di Diegaro, Cesena, Italy). Adenosine 5′-monophosphate sodium salt was purchased from USB Biochemicals, Societa' Italiana Chimici (Rome, Italy). All chemicals used were of the highest analytical grade available.

Enzyme Purification and Enzymatic Asssay—Bull semen samples, usually 200 ml after complete liquefaction, were centrifuged at 600 × *g* for 10 min to sediment spermatozoa. The supernatant was centrifuged at 150,000 × *g* for 1 h and the pellet was extracted with 20 ml of 20 mM Hepes/NaOH buffer (pH 7.5) containing 50 mM NaCl, 50 mM sodium cholate, and 0.1 mM protease inhibitor cocktail (antipain, chymostatin, EDTA and Pefabloc® SC), at 37°C for 5 h. The mixture was then centrifuged at 50,000 × *g* for 30 min and the supernatant was used for the purification of the GPI-anchored 5′-nucleotidase as previously described (*18*). Its purity was checked by SDS-PAGE and silver staining (*19*).

The supernatant from the 150,000 × *g* spin usually contains about 50% of the initial activity determined in bull seminal plasma after removal of the spermatozoa. This enzymatic activity represents the "soluble" 5′-nucleotidase component in bull seminal plasma. The K_m for AMP is about 20 μM and it is similar to that of the GPI-anchored 5′-nucleotidase (11 μM). However, this soluble component is not homogeneous, and we were able to isolate from it very small amounts of the high-K_m AMP- and IMP-preferring forms of 5′-nucleotidase, the low-K_m AMP-preferring enzyme constituting the greatest portion of the soluble 5′-nucleotidase found in bull seminal plasma (results not published). Given this heterogeneity of the soluble 5′-nucleotidase normally occurring in bull seminal plasma, in order to clarify the effect of the GPI moiety on the thermal behavior of the ecto-5′-nucleotidase, we preferred to obtain soluble anchor-less 5′-nucleotidase by removing the GPI anchor from the GPI-containing 5′-nucleotidase through treatment with PI-PLC. In the following, this soluble form of ecto-5′-nucleotidase is referred to as "solubilized ecto-5′-nucleotidase," "solubilized ectoenzyme," or "solubilized enzyme." Solubilized ecto-5′-nucleotidase was obtained by treatment of purified GPI-anchored 5′-nucleotidase with PI-PLC from *B. cereus* (Sigma). GPI-anchored 5′-nucleotidase, usually 1 mg/ml in the Hepes/NaOH buffer, was incubated overnight at 30°C, in the presence of 0.2 unit of PI-PLC. After cooling at room temperature (about 22°C), the mixture was applied to a Sepharose-AMP affinity column (1 × 10 cm, Sigma) equilibrated with 10 mM imidazole (pH 6.8), containing 50 mM NaCl, and 1 mM NaN_3. The column was washed with the buffer and 5′-nucleotidase elution was achieved with 10 mM AMP in the same buffer. 5′-AMPase activity was determined by measuring the amount of inorganic phosphate released in the incubation mixture, which contained 5 to 8 ng of 5′-nucleotidase and variable amounts of the substrate 5′-AMP in 300 μl (final volume) of 50 mM Tris-Cl buffer, pH 7.5. After incubation under the specified experimental conditions, the reaction was quenched by adding 1.7 ml of a solution containing one part of 10% ascorbic acid and six parts of 0.42% ammonium molybdate in 0.5 M sulfuric acid. The mixture was incubated at 45°C for 30 min and the absorbance measured at 820 nm against blanks, according to Chen *et al.* (*20*). In the standard assay, the reaction was carried out at 25°C.

Protein Determination—Protein concentration was determined by using bicinchoninic acid reagent according to Smith *et al.* (*21*), with ribonuclease A and bovine serum albumin as standards.

Enzyme Thermophilicity and Thermostability—Ecto- and solubilized 5′-nucleotidase thermophilicity was tested in the temperature range of 25-85°C. The assay mixture contained, in a final volume of 0.3 ml, 50 mM Tris-Cl pH 7.5, 0.1 mM AMP, and 5.0 ng of the relevant enzyme.

Enzyme thermostability was tested by incubating in sealed Eppendorf tubes 0.2 mg/ml of purified 5′-nucleotidase in 50 mM Tris-Cl buffer, pH 7.5, at 25, 40, 55, 65, and 75°C. At fixed intervals, aliquots of 40 ng of the pre-incubated enzyme were withdrawn from the mixture and the activity was measured under the standard assay conditions.

Fluorescence Spectroscopy—Measurements were performed on homogenous samples at a protein concentration of 0.05 mg/ml. In order to determine only the tryptophan perturbation, the excitation wavelength was fixed at 295 nm. All measurements were performed in the temperature range of 25-95°C on a model FP 777 Jasco spectrofluorimeter by using a 1.0 cm light path fluorimetric quartz cuvette. Spectra were recorded at 0.5-nm intervals, at a scan speed of 20 nm/min, with 5 accumulations. The response was set to 1 s; the excitation and emission bandwidths were 5 and 10 nm, respectively.

Circular Dichroism Spectroscopy—Circular dichroism spectroscopy was performed on homogenous samples, at protein concentrations between 0.2 and 0.5 mg/ml in a 50 mM Tris-Cl buffer, pH 7.5. A model J-710 Jasco spectropolarimeter (Jasco, Tokyo) equipped with a temperature-controlled liquid system Neslab RTE-110 (Neslab Instruments, Portsmouth, NH, USA) was used. Calibration was achieved by means of a standard solution of (+)-10-camphorsulphonic acid. Cuvettes of 0.1 and 1.0 cm path length (Helma, Jamaica, New York, USA) were employed respectively in the far- (190-240 nm) and near-UV (250-300 nm) regions. Photomultiplier absorbance did not exceed 600 V in the spectral regions measured. Each spectrum was averaged five times and smoothed with the Spectropolarimeter System Software Ver. 1.00 (Jasco, Tokyo). All measurements were performed under a nitrogen flow. Results are expressed in terms of molar ellipticity (θ_M).

RESULTS

Thermophilic Behavior—Figure 1 shows the effect of temperature on the V_{max} of the two enzymes. The enzyme activity increases for both enzymes up to 60°C and the Arrhenius plots, drawn as insets, are linear in the temperature range of 25-60°C, suggesting that no marked conformational changes occurred (*22*). The activation energies, calculated from the slopes of the Arrhenius plots, are 44.4±1.9 and 51.8±2.2 kJ/mol for the solubilized and GPI-anchored enzymes, respectively.

The determination of K_m and k_{cat} values for the two enzymes in the temperature range of 25-85°C yielded the pattern shown in Fig. 2. As can be seen, the catalytic efficiency of both enzymes, expressed by the k_{cat}/K_m ratios, decreases steeply in the temperature interval of 25-40°C.

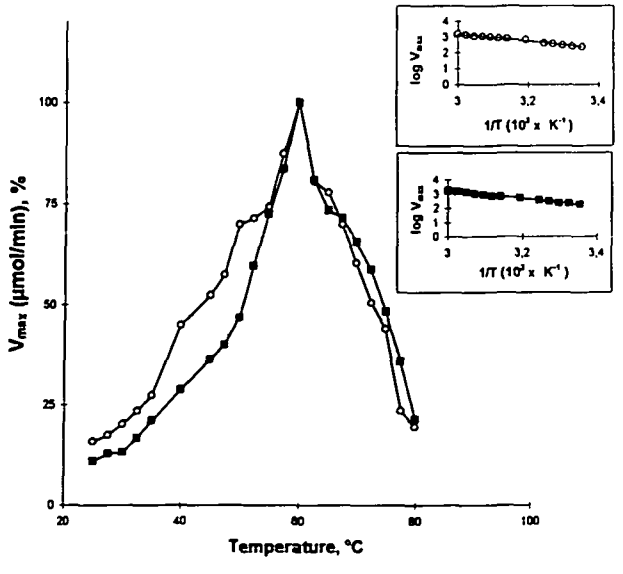

Fig. 1. **The effect of temperature on 5′-AMPase activity of GPI-anchored (filled symbols) and solubilized (open symbols) 5′-nucleotidase.** The Arrhenius plots are shown in the insets. Plots are linear in the temperature range of 25-60°C.

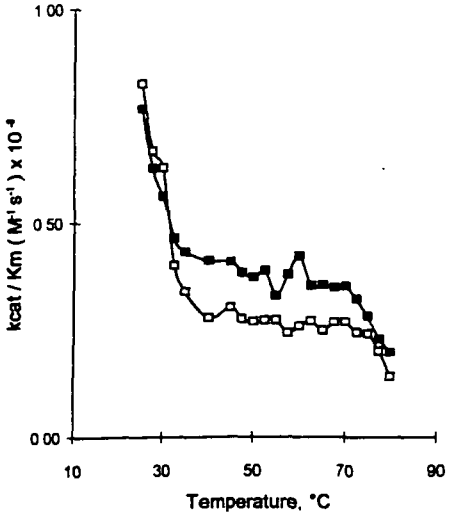

Fig. 2. **The effect of temperature on k_{cat}/K_m ratios for GPI-anchored (filled symbols) and solubilized (open symbols) 5′-nucleotidase.**

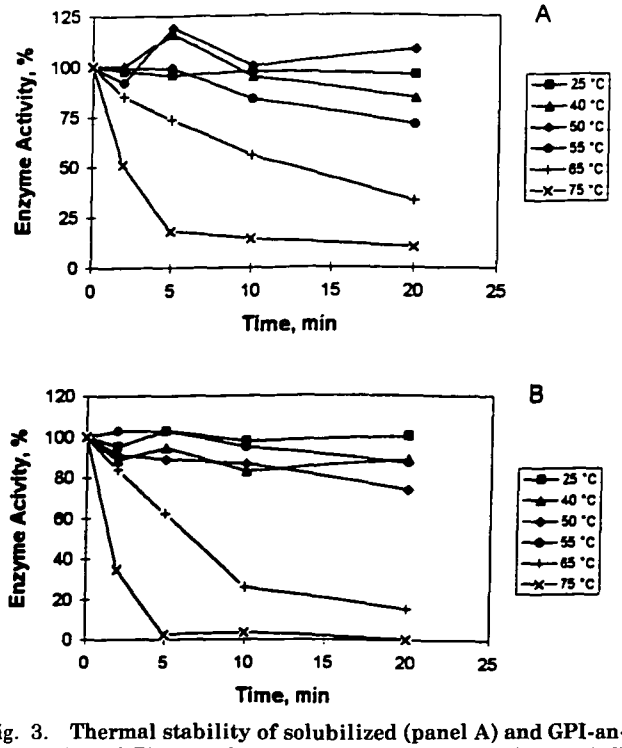

Fig. 3. **Thermal stability of solubilized (panel A) and GPI-anchored (panel B) 5′-nucleotidase.** Enzyme solutions (200 µg/ml) were incubated at the selected temperatures. Aliquots were withdrawn at the indicated times and the 5′-AMPase activity was assayed at 25°C under the standard assay conditions.

Thermostability—The unusual thermophilic behavior shown by the enzyme prompted us to investigate the effect of temperature on the structural properties of the enzyme in order to correlate the functional and structural features.

The effect of pre-incubation at different temperatures on the AMP hydrolysis rate of the enzyme is shown in Fig. 3 (A and B). Preincubation of solubilized 5′-nucleotidase at 40-50°C resulted in an enzyme activation of about 20% (Fig. 3A).

The thermal stability of the ecto-5′-nucleotidase is shown in Fig. 3B. Contrary to what was observed for the solubilized enzyme, pre-incubation at 40-50°C did not induce any enzyme activation. Moreover, in order to determine the deactivation rate constants (k_d) and the half-lives of the two enzymes ($t_{1/2}$), plots of log $[E]_t$ *versus* time at various temperatures were examined. The results are summarized in Table I. As can be seen, at temperatures between 25 and 40°C the solubilized enzyme is significantly more thermostable than the ectoenzyme, while at temperatures higher than 55°C, the difference between the two enzymes leveled off.

Far- and Near-UV Circular Dichroism—The secondary structure of the two enzymes was investigated by far-UV circular dichroism measurements. The CD spectra of solubilized and GPI-anchored nucleotidases at 25°C were superimposible, suggesting that the two proteins possessed the same secondary structure contents (data not shown). The analysis of CD data by means of Yang's algorithm (*23*) produced the percentage fractions of secondary structure shown in Table II. As can be seen, at 95°C both the ecto- and the solubilized 5′-nucleotidase maintain about 70% of the α-helix content and over 80% of the β-sheet structure observed at 25°C.

The near-UV CD spectra of solubilized 5′-nucleotidase are shown in Fig. 4. At 25°C, the spectrum is well structured, suggesting that most of the aromatic residues are constrained by tertiary interactions in compact regions of the folded macromolecule. The minimum at 293 nm and the maximum at 274 nm correspond to the dichroic activity of tryptophan and tyrosine residues, respectively (*24*). Moreover, the presence of the shoulder at 254 nm indicates

TABLE I. **Deactivation rate constant (k_d, min^{-1}) and half-life ($t_{1/2}$, min) values for solubilized ecto- and ecto-5′-nucleotidase from bull seminal plasma.**

Temperature (°C)	Solubilized ecto-5′-nucleotidase		Ecto-5′-nucleotidase	
	k_d	$t_{1/2}$	k_d	$t_{1/2}$
25	1.30×10^{-5}	53,307	2.71×10^{-5}	31,919
40	2.17×10^{-5}	31,139	3.47×10^{-5}	19,950
55	6.08×10^{-4}	1,139	6.08×10^{-4}	1,139
65	5.64×10^{-3}	122	4.73×10^{-3}	146
75	8.50×10^{-2}	8	7.31×10^{-2}	9

TABLE II. **Secondary structure content of solubilized ecto- and ecto-5′-nucleotidase evaluated according to the method of Yang *et al.* 1986.**

Solubilized ecto-5′-nucleotidase		Ecto 5′-nucleotidase
25°C (%)		
α-helix	28	28
β-sheet	55	53
Turn	13	15
Unordered	4	4
95°C (%)		
α-helix	20	21
β-sheet	45	44
Turn	15	14
Unordered	20	21

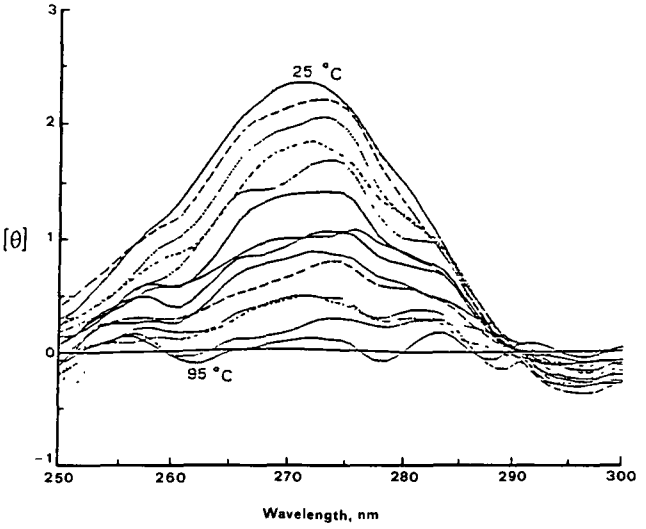

Fig. 4. **Near-UV CD spectra of solubilized 5′-nucleotidase.** Protein concentration: 0.5 mg/ml in 10 mM Tris-HCl, pH 7.0. Spectra were recorded at 5°C increments in the temperature range of 25-95°C.

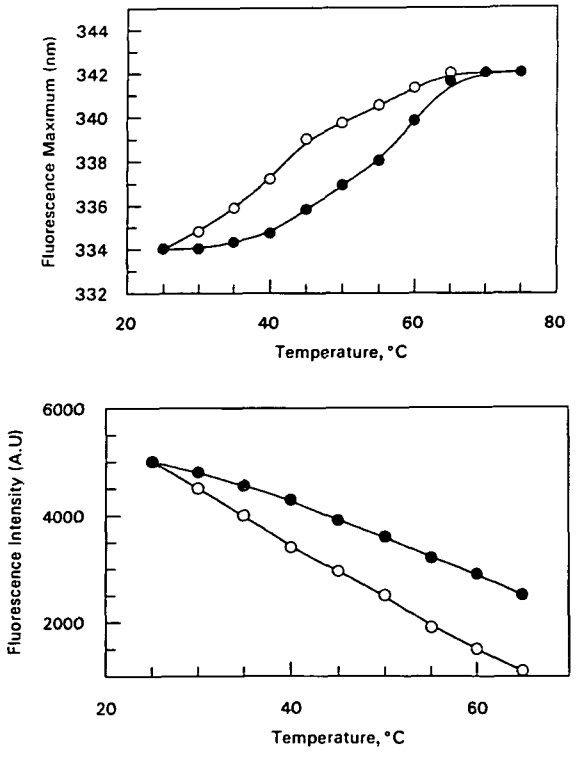

Fig. 5. **Emission maximum position (panel A) and maximal intensity (panel B) of GPI-anchored (filled symbols) and solubilized (open symbols) 5′-nucleotidase.** The excitation wavelength was 295 nm and the protein concentration was 0.05 mg/ml in 10 mM Tris-HCl, pH 7.0.

the contribution of cysteine residues to the CD spectrum. The analysis of the spectra recorded between 25 and 95°C shows that when the temparature is raised the dichroic activity diminishes: in particular, the signals at 274 and 293 are quenched, and at 95°C the three-dimensional structure is completely lost. However, at this last temperature, the shoulder previously observed at 254 nm is converted to a well defined peak, probably related to solvent expulsion by the cysteine residues.

The spectra of the solubilized enzyme and the GPI-anchored enzyme (Fig. 4) showed almost the same pattern.

Fluorescence Spectroscopy—The fluorescence spectra of both ecto- and solubilized 5′-nucleotidase at 25°C show the emission maximum at 334 nm, suggesting that the tryptophanyl residues are partially buried within the protein matrix. Fluorescence spectra performed stepwise at 5°C increments from 25 to 45°C resulted in a 5 nm red-shift of the fluorescence emission maximum for the solubilized enzyme (Fig. 5A). However, when the temperature was raised to 75°C, the emission maximum red-shifted to 342 nm. On the contrary, only a 2 nm red-shift of the emission maximum was observed for the ectoenzyme in the temperature conditions choosen for the solubilized ectoenzyme. Figure 5B shows the the fluorescence intensity dependence on temperature for the two enzymes. Fluorescence intensity of both enzymes decreases as temperature increases, and the fluorescence quenching appears to be more marked for the solubilized ectoenzyme.

DISCUSSION

The results obtained from the comparative thermophilic analysis indicate a role of the GPI anchor in providing the ectoenzyme with a better catalytic efficiency in the temperature range of 25-70°C, while the thermostability analysis suggests an improvement of this property for the solubilized ectoenzyme in the temperature range of 65-75°C. Thus, the presence of the GPI moiety seems to exert a perturbing effect on the structural features of the protein molecule.

The high values (about 10^8 $mol^{-1} \cdot s^{-1}$) observed for the k_{cat}/K_m ratios are very close to the limiting value of 10^8-10^9 $mol^{-1} \cdot s^{-1}$ referred to the virtual condition of optimal catalysis described for other systems, such as carbonic anhydrase (*25*), fumarase (*26*), acetylcholine esterase (*27*), and β-lactamase (*28*). Generally speaking, such high values may be indicative that evolution of the protein is almost completed.

The structural data, gained from the CD spectra, cast light on the thermostable behavior of this mammalian enzyme. In fact, the far-UV circular dichroism spectra showed that temperature increases up to 90°C did not cause any major alteration in the secondary structure content of the solubilized and ecto-5′-nucleotidase, and only at 95°C was a slight decrease of the α-helix content observed. However, as indicated by fluorescence spectroscopy and near-UV circular dichroism, temperature increase affected the tertiary structure of both enzymes. As shown in Fig. 5, the GPI-enzyme is more thermoresistant than the solubilized enzyme, and we could ascribe the greater thermoresistance of the ectoenzyme to the presence of the anchor.

It is noteworthy that the incubation at 30-45°C resulted in 20% activation only of the solubilized ectoenzyme. This could be due to the higher rigidity of the ectoenzyme caused by the presence of the anchor. Moreover, fluorescence spectroscopy depicted a different temperature dependence of the structure. When the temperature was raised to 45°C (see Fig. 5A), a peak shift of 5 nm was observed for the solubilized enzyme, while only a 2 nm shift was observed for the ectoenzyme, and this could reflect differences in the flexibility/rigidity of the two enzymes.

The kinetic data indicate that the presence of the GPI anchor does not modify the geometry of the active center for the AMPase activity; the thermal stability, as well as the catalytic efficiency of the GPI-anchored protein, are well preserved in Tris-Cholate buffers.

The energy of activation values for AMP hydrolysis catalyzed by the anchor-less and the GPI-anchored 5′-nucleotidase are 44.4 and 51.8 kJ/mol, respectively. This difference can be ascribed to different conformational flexibilities of the two enzymes in the temperature range investigated. These values are similar to those described for alcohol dehydrogenase from the thermophilic microorganism *Bacillus acidocaldarius* (42 kJ/mol) (*29*) and β-glycosidase from the extreme thermophilic archaeon *Sulfolobus solfataricus* (47.8 kJ/mol) (*30*). Moreover, the pattern of the enzymatic activity depicted in Fig. 1 is also similar to those observed for the two thermophilic enzymes. However, a remarkable difference between the 5′-nucleotidase from bull seminal plasma and enzymes from moderate or extreme thermophiles is that bull seminal plasma 5′-nucleotidase also shows high activity at 20°C, whereas enzymes from thermophilic microorganisms are almost inactive up to 40°C. A thermostability investigation on D-glyceraldehyde-3-phosphate dehydrogenase from lobster muscle, a mesophile, *Bacillus stearothermophilus*, a moderate thermophile, and *Thermus aquaticus*, an extreme thermophile, showed that protection of the enzyme activity against thermal denaturation increased on going from the mesophile to the extremophile and the thermostability was closely related to the rigidity of the protein structure (*31*). The expression of the enzymatic activity depends on a compromise between the need for sufficient structural flexibility for full activity and the need for structural rigidity to protect the enzyme from inactivation. The result is that mesophile enzymes are normally active between 20 and 50°C, while thermophile enzymes are normally active between 40 and 90°C or even above. In this context, the present study indicates that 5′-nucleotidase from bull seminal plasma combines the properties of mesophile and moderate thermophile enzymes, with the GPI-anchored form showing better thermostability.

This offers interesting perspectives in view of the *in vitro* conversion of GPI-non-containing into GPI-anchored proteins, which, in turn, could easily be incorporated into matrices of various types with an extra-membrane localization of the protein moiety. In a previous paper (*32*) we have shown that the ecto-5′-nucleotidase from bull seminal plasma could be reconstituted in electron spin-labeled hen egg yolk lecithin liposomes, with the dimeric protein moiety containing both the AMPase activity and the site for the PI-PLC action localized at the exterior of the liposome membrane. The attachment of GPI anchors to proteins could be included in the list of techniques available for the design of new biocatalysts required by modern organic synthesis and general biotechnological applications.

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