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Spectroscopic study of fluorescent peptides for prenyl transferase assays

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Abstract

A study of the prenyl transferase reactions was performed by fluorescence using rat brain cytosol fractions as an enzyme source. Four dansylated peptides corresponding to the C-terminal sequence of Ras isoforms were synthesised. The effects of different detergents on the farnesylation or geranylgeranylation of the four peptides were evaluated. Dose-dependent effects of dodecyl-maltoside, a non-ionic detergent, on the farnesyl transferase or geranylgeranyl transferase activities were observed with all peptide substrates. Additionally, the effect of temperature was investigated and these assays were applied to determine Michaelis–Menten constants (K_m) of the substrates: dansyl-GCVLS (1.8 μM), dansyl-GCVVM (3.2 μM), dansyl-CVIM (3.4 μM) and dansyl-GCVLL (8.4 μM) and FPP (22.6 μM) for FTase activity. Using GGPP as co-substrate, GGTase activity was measured with K_m values superior to 50 μM for all the three substrate dansyl-GCVLS, dansyl-GCVVM, or dansyl-CVIM, whereas values of 7.6 and 5.4 μM were calculated for the dansyl-GCVLL sequence and GGPP co-substrate, respectively.

IC_{50} values of selective prenyl transferase inhibitors, B-581, FTI 276 and GGTI 287 have been measured to 34, 0.8 and 18 nM, respectively, using dansyl-GCVLS as substrate (FTase inhibition). When dansyl-GCVLL is used as substrate (GGTase inhibition) the IC_{50} values are 5100, 75 and 5 nM for B-581, FTI 276 and GGTI 287, respectively. Then, this developed method allowed to evaluate the selectivity of all the three inhibitors tested.

Keywords: Dansyl peptides; Fluorescence spectroscopy; Farnesyl transferase; Geranylgeranyl transferase; *n*-Dodecyl-β-D-maltoside; Cytosolic enzymes

1. Introduction

Major components of the small G protein family are the Ras proteins including H-Ras, N-Ras and K-Ras 4A/4B [1]. These proteins require a membrane localisation to express their functional activity. The post-translational protein modifications are achieved by farnesyl transferases (FTase) which transfer a 15-carbon farnesyl group to a cysteine at the carboxyl terminal sequence of Ras, called the CAAX box where C is Cys, A is an aliphatic amino-acid, X is Met, Ala, Gln or Ser residue [2]. Other homologous prenyl transferases are the geranylgeranyl transferases (GGTase), GGTase I and II. Substrates of GGTase I include a variety of G proteins such as Rap 1A, Rac 1 and the γ-subunit of heterotrimeric (α, β, γ) mammalian G-proteins. GGTase I catalyses the transfer

Abbreviations: CMC, critical micelle concentration; DM, *n*-dodecyl-β-D-maltoside; DTT, dithiothreitol; FMOC, fluorenylmethoxycarbonyl; FPP, farnesyl pyrophosphate; FTase, farnesyl transferase; GGPP, geranylgeranyl pyrophosphate; GGTase, geranylgeranyl transferase; K_m, apparent Michaelis-Menten constant; OGP, *n*-octyl-β-D-glucopyranoside

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of the C-20 geranylgeranyl group to the cysteine residue of a CAAL box. GGTase II is responsible for the addition of geranylgeranyl group to proteins such as Rab that have the consensus sequence CC or CXC at the carboxyl terminus [3].

Ras proteins are implicated in the signal transduction of growth factors and mutations of Ras occur in 20–30% of all human cancer cells [4]. Prenylation prevention precludes membrane attachments and abolishes the malignant transforming ability of oncogenic H-Ras [5]. Thus, inhibition of Ras prenylation represents an important strategy for the treatment of cancer [6]. Synthetic compounds based upon the structure of naturally-occurring peptide substrate have been designed as inhibitors for FTase [7–9]. Recent clinical data have shown that this strategy of targeting Ras function in cancer cells does not need to be limited to tumours with proven oncogenic Ras mutation [10].

Several studies used [^{3}H]FPP or [^{3}H]GGPP to perform FTase and GGTase assays [11–13] but it would be convenient to develop a sensible and flexible method not using radiolabelled substrates. Fluorescence experiments based on spectrofluorimetric properties of a dansyl moiety linked to a peptidic substrate sequence were reported using human recombinant enzymes hFTase [14] and hGGTase I [15] and dansyl-GCVLS and dansyl-GCVLL, as selective substrates, respectively.

In the present paper, this fluorescence assay was employed with rat brain cytosol as an enzyme source [16]. Three peptidic sequences, substrates of FTase (such as H-Ras, N-Ras and K4A-Ras) dansyl-GCVLS, dansyl-GCVVM, and dansyl-GCVIM (or dansyl-CVIM) were tested. In parallel, the dansyl-GCVLL peptide was used as a substrate in geranylgeranyl transferase reaction. The fluorescence response of each peptide, particularly sensitive to the physicochemical parameters of the medium, was optimized and the influence of specific detergents, and the temperature were studied. Michaelis constants (K_m) for all peptides, inhibition constants (IC_{50}) and selectivity of reference inhibitors were measured.

2. Materials and methods

2.1. Chemicals

n-Dodecyl-β-D-maltoside (DM), *n*-octyl-β-D-glucopyranoside (OGP), deoxycholic acid (ULTROL® grade), Triton® X-100 (Protein grade®) and CHAPS were purchased from Francebiochem (Meudon, France). Dansyl-glycine, dithiothreitol (DTT), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) were obtained from Sigma (St. Quentin Fallavier, France). Peptides were prepared by standard solid-phase synthesis methods using FMOC (fluorenylmethoxycarbonyl) chemistry. Peptides were dansylated according to a procedure described by Pompliano et al. [14]. Purity controls were achieved by HPLC and mass spectrometry analysis (97.5–99% purity range). Concentration of dansylated peptides were determined by absorbance measurements at 340 nm. Dansyl-glycine was used as a reference standard for calibration, as previously described [17].

2.2. Preparation of cytosolic fractions

Male Sprague–Dawley rats (9–10 weeks old) were obtained from Charles River (St. Aubin les Elbeuf, France).

The animals were sacrificed according to the Institutional Protocol [16]. The brain was removed and immediately placed in ice cold 0.1 M HEPES buffer, pH 7.4, containing 25 mM $MgCl_2$, and 10 mM DTT. The tissue was cleaned thoroughly, minced and homogenized in the same buffer with a polytron (ten burst at 800 rpm). The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C. The resulting supernatant was then centrifuged again at 100,000 × *g* for 60 min at 4 °C and the resulting supernatant (the cytosolic fraction) was aliquoted to 1 ml vials and stored at −80 °C. The protein concentration was calculated by the method of bicinchoninic acid (BCA) (Interchim, Monluçon, France) using bovine serum albumin as the reference standard.

2.3. Prenyl transferase assays

FTase activity was carried out as follows unless otherwise stated: the mixture containing 30 μg of cytosolic protein (250 μl in eppendorf tube), the dansyl peptide (1.6–6 μM), and the detergent (see optimal concentrations) in a 52 mM Tris–HCl buffer pH 7.4 supplemented with 5.0 mM DTT, 12 mM $MgCl_2$, 12 μM $ZnCl_2$ was incubated at 30 °C for 2 min at a constant temperature before the reaction was initiated by the addition of 25 μM FPP. The contents were quickly mixed by flicking the tube and a 200 μl sample was pipetted into the prewarmed cuvette. Quartz cuvettes (3 mm square) were purchased from Hellma (Paris, France). The fluorescence intensity was recorded, at a fixed excitation and emission wavelengths 340 and 530 nm, respectively, for 750 s. Rates (counts per second per second, cps/s) were measured from the initial linear regions of each run and all measurements were made in duplicates of three independent experiments.

Endogenous fluorescence from rat brain cytosol provoked a basal increase of the fluorescence intensity at 530 nm (10% from the initial value), which remained constant without peptide addition.

GGTase activity was assayed by a similar method but substituting the substrates by 4 μM dansyl-GCVLL and 6 μM GGPP.

Data were collected on a Spex Fluoromax spectrofluorimeter (ISA-HORIBA, Longjumeau, France). For all dansylated peptides, the excitation wavelength was 340 nm (slit width = 10 nm), and the emission wavelength was 505 nm (slit width = 10 nm).

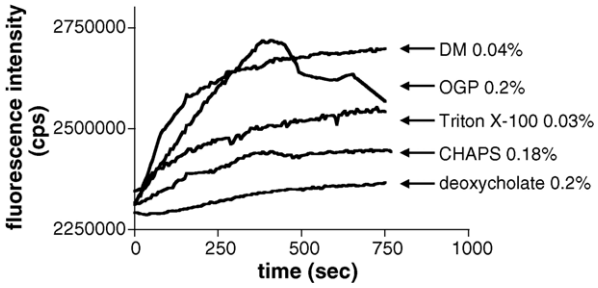

Fig. 1. Time-dependent variation of fluorescence intensity upon farnesylation of dansyl-GCVLS in the presence of various detergents.

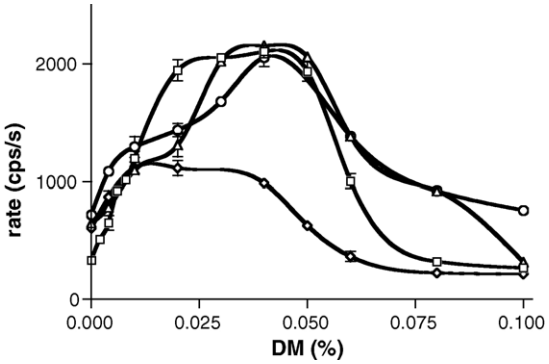

Fig. 2. Variation of the DM concentration on the initial reaction rate (cps/s) of dansyl peptide farnesylations ((□) dansyl-GCVLS, (△) dansyl-GCVVM and (◯) dansyl-CVIM in the presence of FPP) or dansyl-GCVLL (◊) geranylgeranylation in the presence of GGPP.

3. Results

3.1. Prenylation reaction of different peptide sequence

Four representative peptides of the Ras isoform were studied by the fluorescence assay. We have recently shown that farnesylation and geranylgeranylation can be performed with the dansylated peptides GCVLS and GCVLL, respectively [9]. Additionally, here dansyl-GCVVM and dansyl-GCVIM were chosen as substrates of farnesyl transferase corresponding to different isoforms of Ras protein. We observed a variation of fluorescence intensity at 530 nm between 2,250,000 and 2,700,000 cps using dansyl-GCVVM but surprisingly, the fluorescence intensity of the related peptidic dansyl-GCVIM sequence, which exhibits the same spectral properties, did not increased significantly in the presence of FPP or GGPP and rat brain cytosolic proteins used as the enzyme source. A previous report described a number of other dansylated peptides for this fluorescence assay. Among them, dansyl-CVIM showed a significant increase of fluorescence intensity [14]. This peptidic sequence is then synthesised for further investigation. The farnesylation of dansyl-CVIM is observed by a fluorescence increase at 530 nm between 2,250,000 and 2,700,000 cps as observed for the three other peptidic sequences.

3.2. Detergent variation

As described by several studies using a real-time fluorescence assay for protein prenyl transferases [14,15,17], the assay is particularly sensitive to the nature and amount of detergent used. To address this issue, we studied effects of various detergents on the prenylation of four dansyl peptide sequences. For the kinetic measurements, the reactions are performed at saturating concentrations of substrates and the detergent concentrations are chosen near the critical micelle concentration (CMC) [18]. Fig. 1 shows typical continuous fluorescence measurements in the presence of dansyl-GCVLS and several specific detergents. Similar curve profiles are recorded with all the other dansylated peptide sequences. Activities of farnesyl transferase and geranylgeranyl transferase are reported in Table 1. *n*-Dodecyl-β-D-maltoside (DM), *n*-octyl-β-D-glucopyranoside (OGP), and Triton® X-100 gave the best satisfactory initial velocity of reaction for all peptides. The progress curves are smooth but OGP did not provide a curve with a stable maximum. OGP at 0.2% gave an irregular progress curve. The detergent does not stabilise the fluorescence signal of the prenylated product, as illustrated by a fluorescence decrease after reaching a maximum. CHAPS and deoxycholic acid, at concentrations far above the CMC, gave a reduced fluorescence enhancement. Rates of fluorescence intensity are also smaller with these detergents than with non-ionic detergents (Table 1). The velocity value is reduced four or seven times when using CHAPS or deoxycholic acid, respectively. The effect of various detergents is less pronounced with dansyl-GCVLL, substrate for geranylgeranyl transferase. In this case, DM 0.04%, OGP 0.2% and Triton® X-100 0.03% gave same satisfactory progress curves and the discrepancy of velocities between non-ionic and zwitterionic or anionic detergents are reduced (Table 1).

The CMC value of DM is given from 0.005 to 0.03% (temperature 20–25 °C) [18]. Prenylations of the four dansyl peptides are evaluated in the presence of increasing DM concentrations (Fig. 2). Without detergent, the rates of increase in fluorescence intensity are quite similar for all peptides.

Table 1
Influence of detergents on the initial reaction rate (cps/s) for dansyl peptide prenylation

	DM (0.04%)	OGP (0.2%)	Triton® X-100 (0.03%)	CHAPS (0.18%)	Deoxycholic acid (0.2%)
Dansyl-GCVLS	2170 ± 90	1128 ± 32	1252 ± 25	499 ± 22	274 ± 13
Dansyl-GCVVM	2114 ± 57	1193 ± 35	1198 ± 19	391 ± 24	280 ± 10
Dansyl-CVIM	2094 ± 132	1302 ± 39	1229 ± 28	564 ± 23	262 ± 11
Dansyl-GCVLL	985 ± 44	1070 ± 34	967 ± 35	414 ± 55	252 ± 10

The fluorescence signal is a little bit noisy because fluctuations are observed during the reaction. Farnesylations of dansyl-GCVLS, dansyl-GCVVM, or dansyl-CVIM are enhanced by the presence of DM. A maximum rate of increase in fluorescence intensity is reached at 0.02% DM for dansyl-GCVLS, whereas optimal farnesylations of other dansyl peptides are recorded in the presence of higher DM concentrations. Initial reaction rates of farnesylation of the four dansyl peptides are constant in a limited DM concentration range. The largest range is recorded for dansyl-GCVLS (from 0.02 to 0.05%), whereas for dansyl-GCVVM and dansyl-CVIM, the range are from 0.03 to 0.05% and from 0.04 to 0.05%, respectively.

Dansyl-GCVLL (in the presence of GGPP) is geranylgeranylated with a rate of fluorescence increase lower than for farnesylation (Table 1). The effect of the DM concentration is less pronounced than for farnesylation of other peptides. The range for optimal progress curves is from 0.01 to 0.04%. For all peptides, concentrations of DM over 0.05% decrease sharply the initial velocity of the prenylation reactions.

3.3. Temperature variation

The effects of temperature on the FTase and GGTase activity assays are shown in Fig. 3. The variation from 20 to 30 °C causes a rapid increase of FTase activity (two times higher value), whereas GGTase activity is constant from 20 to 40 °C. Stable values of FTase activity are observed in the 30–40 °C range. Higher temperatures such as 50 or 60 °C reduces FTase and GGTase activities by 50 and 34%, respectively. Experiments of enzyme denaturation are performed at 100 °C for 30 min. Identical runs as described above were used in the optimal conditions for prenylation. In the presence of FPP or GGPP, progress curves are recorded without significative fluorescence variations. Weak slopes, corresponding to the background noise, are calculated to 15.3 ± 3.4 cps/s. Thus, a significative rate of increase in fluorescence is determined as ten times this value, i.e. 153 cps/s.

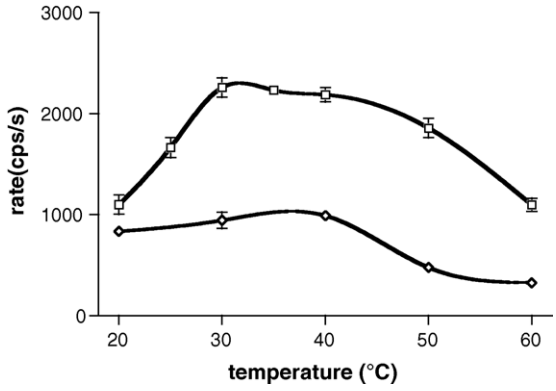

Fig. 3. Variation of temperature for continuous fluorescence measurements on farnesylation of dansyl-GCVLS (□) and geranylgeranylation of dansyl-GCVLL (◊) with DM 0.04%.

3.4. Activity measurements

These assays are particularly well suited for kinetic measurements. Apparent binding constant determinations are performed under optimal conditions, using 0.04% DM and at 30 °C.

In the farnesylation procedure, the fluorescence velocity is linearly dependent upon enzyme concentration. Linear regression, performed in the concentration range of 0.01 to 0.125 g L^{-1} (six concentrations in triplicate), shows a slope value of $1.54 \times 10^4 \pm 9.24 \times 10^2$ cps L s^{-1} g^{-1} and a intercept of $2.92 \times 10^2 \pm 1.31 \times 10^1$ cps s^{-1} ($r = 0.976$).

Apparent Michaelis–Menten constants, K_m, of dansyl peptides and FPP were calculated (Table 2). The values for dansyl-GCVLS, ($K_m = 1.8 \pm 0.3$ μM) and FPP, ($K_m = 22.6 \pm 4.9$ μM in the presence of dansyl-GCVLS), are in agreement with the literature [14]. Although there is no published K_m for dansyl-GCVVM or dansyl-CVIM, values obtained here are similar to the Michaelis–Menten constant of a related peptide sequence, dansyl-GCVIA (2 μM) [17]. Additionally, dansyl-GCVLL is farnesylated in the presence of FPP and FTase as reflected by a K_m value of 8.4 ± 0.8 μM.

The fluorescence assay for geranylgeranylation of dansyl-GCVLL was performed in the presence of GGPP. The reaction was linear with respect to the enzyme concentration (slope = $8.34 \times 10^3 \pm 2.50 \times 10^2$ cps L s^{-1} g^{-1} and intercept = $1.25 \times 10^2 \pm 3.12$ cps s^{-1}; $r = 0.988$). The K_m for dansyl-GCVLL was 7.6 ± 0.8 μM and for GGPP it was 5.4 ± 1.4 μM in the presence of dansyl-GCVLL. Peptides corresponding to farnesylation substrates are tested for geranylgeranylation activity in the presence of GGPP and rat brain cytosolic proteins as the enzyme source. In agreement with Pickett et al. [15], dansyl-GCVLS is found to be completely inactive. Dansyl-GCVVM and dansyl-CVIM are substrates for geranylgeranylation but the rates of increase in fluorescence intensity are low, i.e. significant rates were measured for concentrations > 50 μM.

3.5. Application for testing of inhibitors

Inhibition of prenyl transferases is also investigated. Four peptides are used as substrates to evaluate the effects of reference inhibitors (Fig. 4) including B-581, FTI 276, and GGTI 287 [7,8]. No interference by undesired fluorescence was measured for these compounds. Experimental inhibition

Table 2
Michaelis–Menten constants of dansyl peptides in the presence of FPP (farnesylation) or GGPP (geranylgeranylation)

	FTase activity K_m (μM)	GGTase activity K_m (μM)
Dansyl-GCVLS	1.8 ± 0.3	> 100
Dansyl-GCVVM	3.2 ± 0.5	> 50
Dansyl-CVIM	3.4 ± 0.4	> 50
Dansyl-GCVLL	8.4 ± 0.8	7.6 ± 0.8
FPP	22.6 ± 4.9	–
GGPP	–	5.4 ± 1.4

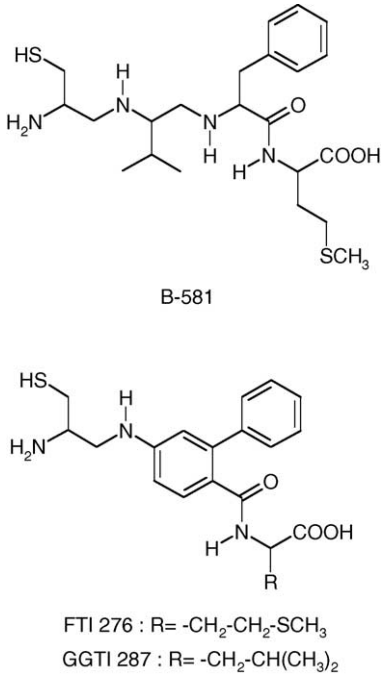

Fig. 4. Structures of the B-581, FTI 276 and GGTI 287 prenyl transferase.

constant values are reported in Table 3. According to published values for reference inhibitors in different testing systems, IC_{50} measured in these assays show similar inhibitions of FTase or GGTase activities. The known GGTase selective compound B-581 is characterized here with an IC_{50} for GGTase 150 times higher than for FTase. FTI 276 and GGTI 287 are less selective inhibitors than the parent compound. Farnesylations performed with different dansyl peptides are inhibited to the same extend by reference inhibitors, suggesting that IC_{50} values are independent of the peptide sequence.

4. Discussion

In the present study, cytosolic rat brain fractions were used to develop FTase and GGTase assays. This source of enzyme was chosen because most of the enzymology and inhibitor studies should better be performed starting with total cellular proteins [7,8,16]. A strategy of continuous fluorescence measurement using selective dansylated peptides as substrates for both enzymes was successfully developed. Optimal conditions were determined by testing experimental parameters in particular the peptidic sequence, the chemical nature and the concentration of detergent, and the reaction temperature. Additionally, we showed here that the fluorescence method must not be used to measure prenylation of dansyl-GCVIM peptide which is the C-terminal sequence of K-Ras isoform. Because this prenylation reaction is successfully detected with the shorter sequence dansyl-CVIM, we hypothesised that peptidic conformation of dansyl-GCVIM is unfavourable for the enzymatic thioether addition or does not dispose favorably dansyl and farnesyl groups in a close environment. Previous studies have highlighted the key influence of detergents for specific peptide sequence. For example, dodecyl-maltoside is required for optimal farnesylation of dansyl-GCVIA and detergents such as Triton® X-100 or CHAPS are particularly well adapted when using the FTase enzyme [17]. Other peptide sequences, dansyl-GCVLS, dansyl-GCVLL were prenylated in the presence of octyl-β-D-glucopyranoside as a detergent [14,15].

Table 3
IC_{50} values for B-581, FTI 276 and GGTI 287 prenyl transferase inhibitors in the presence of the dansylated peptides GCVLS, GCVVM, or CVIM and FPP, substrates for FTase or in presence of dansyl-GCVLL and GGPP substrates for GGTase

	IC_{50} (nM)		
	B-581	FTI 276	GGTI 287
Dansyl-GCVLS	34 ± 2.1	0.8 ± 0.1	18 ± 1.2
Dansyl-GCVVM	28 ± 3.0	0.8 ± 0.2	22 ± 1.3
Dansyl-CVIM	36 ± 1.8	0.9 ± 0.2	27 ± 1.5
Dansyl-GCVLL	5100 ± 321	75 ± 5.1	5 ± 0.2

The enzyme preparation used in the present study may modify significantly the influence of the detergent depending on the viscosity of the assay solution and protein–protein interactions must be considered as well. As shown here, non-ionic, zwitterionic or anionic detergents have significant effects on prenylation of the different dansyl peptide sequences. A previous study using dansyl-GCVIA, and purified yeast protein farnesyl transferase in the presence of DM showed fluorescence intensity between 250,000 and 1,000,000 cps [17]. In the same conditions (same equipment and identical experimental settings), with all dansylated peptides the values were from 2,200,000 to 2,700,000 cps with the same rate of increase in fluorescence, near 2000 cps/s. The difference of fluorescence range is probably explained by the different enzyme sources. In our conditions, optimal prenylations are strictly dependent on the nature of the detergent and its concentration but the same progress curve profiles were recorded whatever the dansyl peptide used as a substrate. The detergent has little or no effect on the peptide itself but strongly influences the environment of the dansyl moiety within the micelles. The intensity of fluorescence emission was from 1.5 to 5 times greater in the presence than in the absence of detergents and the velocity of prenylation was dependent on detergent concentrations. Without detergent, a rate of increase in fluorescence is observed but the progress curve is not satisfactory because the fluorescence signal of product increases to a maximum and then decreases dramatically. These observations suggest that the detergent allows to stabilise the fluorescence signal over long incubation times. The velocity of the reaction increases with zwitterionic and anionic detergents such as CHAPS and deoxycholic acid, respectively, with a low magnitude compared to the non-ionic detergent. A previous study showed that CHAPS (0.18%) is acceptable for use with purified FTase and dansyl-GCVIA but higher concentrations inhibit the enzyme [17]. In our conditions, the weak influence of CHAPS could be attributed to

an inhibition of the FTase. Deoxycholic acid is the less advised detergent for these assays with initial reaction rates far above the limit of quantification. Alkyl glycosides such as DM and OGP have become more popular as non-ionic detergents to isolate membrane proteins [19]. For prenylation assays, best results were obtained for all dansyl peptides tested with this class of non-ionic detergents. The homogeneity with respect to their composition and structure led to stable interactions and fluorescence signals. Reactions performed at increasing temperatures from 20 to 60 °C show that the maximum activities of both enzymes were attained in the temperature range of 30–40 °C. Activities are rapidly reduced by a temperature exceeding 40 °C. The use of heat denatured enzymes allows to determine a limit of quantification for the rate of fluorescence signal. This calculated value is 153 cps/s. Finally, these assays have been applied to screen selective inhibitors. The data reported here for the reference inhibitors are in good agreement with previous values [7,8]. IC_{50} values of farnesyl transferase inhibitors are not modified in presence of varied dansyl peptides as substrates suggesting that different C-terminal sequences of Ras isoforms are not distinguished by FTase. Moreover, we showed that dansyl-GCVLL, a substrate used for measuring geranylgeranyl transferase activity, exhibits farnesylation with a K_m of 8.4 ± 0.8 μM. However, GGTase is a more selective enzyme than FTase. The dansylated peptides used for GGTase activity rank in the order dansyl-GCVLL > dansyl-GCVVM, dansyl-CVIM ≫ dansyl-GCVLS.

5. Conclusion

In conclusion, we have developed a fluorescence assay for FTase and GGTase in presence of four dansyl peptide substrates. Significant detergent and temperature effects were characterized for prenylations using a rat brain cytosolic fraction as an enzyme source. The best results were obtained with 0.04% dodecyl-maltoside at 30 °C for all peptides. With these optimal conditions, prenyl transferase inhibitors showed similar IC_{50} values whatever the peptide used as a substrate. This rapid and highly sensitive fluorescent assay should be advantageous compared to conventional methods using radioactive substrates such as [^{3}H]FPP or [^{3}H]GGPP. The facile preparation of the enzyme material, mimicking an in vivo situation, reinforces the utility of this convenient and flexible method. This method will be suitable for practical screening of potential inhibition compounds after adjustment of the conditions from the single cuvette to multiple assays (on 96-well format).

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