Primary sequence requirements for *S*-acylation of β_2-adrenergic receptor peptides

Charlène Bélanger, Hervé Ansanay, Riad Qanbar, Michel Bouvier*

Département de Biochimie and Groupe de Recherche sur le Système Nerveux Autonome, Université de Montréal, C.P. 6128, succursale Centre-Ville, Montréal, QC, Canada H3C 3J7

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Abstract Palmitoylation is a post-translational modification that occurs on selected cysteines of many proteins. Since a high proportion of basic and hydrophobic residues is often found near the palmitoylated cysteine, the role of these residues in the selection of specific palmitoylation sites was assessed. Short peptides derived from the β_2-adrenergic receptor sequence, modified to present different proportions of basic, acidic and hydrophobic residues, were tested in an in vitro *S*-acylation assay. Basic residues proved to be essential, whereas hydrophobic residues greatly enhanced *S*-acylation and acidic residues inhibited it. Taken together, these results show that short peptides contain the required molecular determinants leading to selective *S*-acylation. Whether or not these sequence characteristics also contribute to the selectivity of palmitoylation in vivo will need to be further investigated. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Palmitoylation; Acylation; Post-translational modification; Palmitoyl-CoA; G protein-coupled receptor; β_2-Adrenergic receptor

1. Introduction

Palmitoylation is a post-translational modification that occurs on a subset of cellular proteins [1]. This modification involves the formation of a covalent thioester bond between a C_{16} fatty acid and a single cysteine [2]. Unlike myristoylation, farnesylation and prenylation, that are stable co-translational lipid modifications, palmitoylation is post-translational and reversible [3]. Several members of the G protein-coupled receptor (GPCR) family have been shown to be palmitoylated. GPCRs possess seven transmembrane α-helices and play important roles in the detection and transduction of external stimuli, such as light, taste, odor, neurotransmitters and hormones [4]. The abolition of their palmitoylation site by site-directed mutagenesis has been shown to either decrease coupling to G proteins [5], affect receptor internalization [6] or modulate receptor phosphorylation by regulatory kinases [7].

Mechanisms responsible for the palmitoylation process and its specificity are not well understood. Several studies revealed that palmitoylation could occur in vitro on the same sites that become acylated in vivo, in the absence of any specific acyltransferase, using palmitoyl-coenzyme A (CoA) as the acyl donor. This type of reaction, referred as non-enzymatic, pseudoenzymatic or autocatalytic palmitoylation, uncatalyzed transacylation or *S*-acylation was reported for purified myelin proteolipid protein (PLP) [8,9], myelin glycoprotein Po [8,10], rhodopsin [11,12], Semliki Forest virus E2 glycoprotein [13], SP-C surfactant protein [14,15], several G protein α-subunits [16–18], SNAP-25 [19], c-Yes protein tyrosine kinase [20] and protein kinase C [21]. It has also been reported for short peptides derived from the palmitoylation site of myelin glycoprotein Po [10], the carboxy terminus of N-ras [22], the Yes protein tyrosine kinase [20] and for a variety of synthetic cysteine-containing lipopeptides [23]. The labeling of those peptides with [^{3}H]palmitate was dependent on time, concentration of both reactants, temperature and pH.

Protein sequence alignment of several GPCRs revealed the presence of a greater proportion of hydrophobic/basic residues in the vicinity of palmitoylated cysteine(s). However, no strict consensus sequence could be formally identified, we hypothesized that these basic and hydrophobic amino acids may play an important role in promoting and directing the in vitro *S*-acylation. Thus, the main objective of this study was to assess the role of the amino acids neighboring the palmitoylated cysteine in *S*-acylation. For this purpose, we systematically substituted the amino acids flanking the palmitoylated cysteine within peptides derived from the β_2-adrenergic receptor (β_2-AR) sequence (amino acids 337–344) (Table 1). Our results revealed that basic and hydrophobic amino acids next to the palmitoylated cysteine play a crucial role in the in vitro *S*-acylation process, most likely by favoring peptide interaction with the CoA polar head and the acyl chain of the palmitoyl-CoA. These results suggest that some molecular requirements for specificity of the β_2-AR palmitoylation are located within the primary sequence.

2. Materials and methods

2.1. Peptide preparation

The 13 peptides listed in Table 1 were synthesized using *N*-α-fmoc using LKB Biolynx apparatus and purified by HPLC (Waters) using a C18 reversed-phase column (10 μm, 125 Å). The identity of the peptides was verified by mass spectrometry. Lyophilized peptides were dissolved in 0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH (Boehringer Mannheim, Mannheim, Germany) containing 5 mM dithiothreitol (DTT) pH 7.4 and 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich, Oakville, Canada) to their stock concentration of 10 mM.

*Corresponding author. Fax: (1)-514-343 2210.
E-mail: bouvier@bch.umontreal.ca

Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; CoA, coenzyme A; TLC, thin layer chromatography; β_2-AR, β_2-adrenergic receptor; GPCR, G protein-coupled receptor

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2.2. Cell-free S-acylation assay

[^{3}H]palmitoyl-CoA was synthesized as previously reported [10,16]. By this procedure, 50% of the [^{3}H]palmitic acid (NEN, Boston, MA, USA) was converted to its CoA derivative. Standard conditions for *S*-acylation are as follows: peptide (0.2 mM) and [^{3}H]palmitoyl-CoA (125 μCi; ~40 μM) were incubated at 37°C in 0.1 M MOPS–NaOH pH 7.4 with 1 mM DTT in a total volume of 30 μl for the indicated times. The concentration of palmitoyl-CoA used in the assay is comparable to its estimated total cellular concentration but superior to its estimated free concentration [24]. The concentrations of both the peptides and palmitoyl-CoA used in the assays were selected to lie within the linear range of the reaction (data not shown). In order to assess the thioester nature of the link between palmitate and the peptide, products were incubated for 1 h at 37°C with 1 M hydroxylamine pH 7.4 (Sigma-Aldrich, Oakville, Canada). In some experiments, the effect of pH on the in vitro palmitoylation was tested using different buffer solutions: 0.1 M 2-[*N*-morpholino]ethanesulfonic acid (MES) pH 5.5 or pH 6.5, 0.1 M MOPS–NaOH pH 7.0, pH 7.4 or pH 7.9, 0.1 M Tricine pH 8.2 and 0.1 M Tris[hydroxymethyl]-aminomethane hydrochloride (TRIZMA) pH 9.0 (Sigma-Aldrich, Oakville, Canada). Following the incubation period, samples were prepared for thin layer chromatography (TLC) using silica gel 60 plates (Alltech, Deerfield, USA). Separation was carried out using *n*-butanol:pyridine:acetic acid:water (Laboratoire Mat, QC, Canada); 45:30:9:36 by volume, for QEEECERR and QEQQCQRR; 60:30:5:15 by volume, for QELLCLRR, QELLSLRR, QELYCWRR, QQLLCLRR and QAAACARR; 70:30:5:20 by volume for QRRRCRRR to QELLCLQQ) as the ascending solvent systems. Specific solvents for TLC development were selected depending on the polarity of the different peptides used. [^{3}H]palmitate incorporation was detected by autoradiography (3–7 days exposure of the TLC plates). Bands on the autoradiograms were identified by comparing autoradiographic profiles obtained for [^{3}H]palmitate alone, [^{3}H]palmitoyl-CoA synthesis product and palmitoylated peptides. Incorporation of [^{3}H]palmitate was quantified by densitometric analysis.

3. Results

3.1. In vitro acylation of β_2-AR-derived peptide

In an effort to identify the molecular determinants of *S*-acylation selectivity, an easy and rapid method utilizing in vitro acylation of peptides was selected. First, an octapeptide corresponding to the amino acids 337–344 from β_2-AR sequence (QELLCLRR) was synthesized. Incubation of this peptide with [^{3}H]palmitoyl-CoA for 120 min at 37°C lead to the incorporation of [^{3}H]palmitate into the peptide (Fig. 1A). Identification of the products was confirmed using labeled synthetic palmitoyl-CoA and palmitate (data not shown). No palmitoylated peptide band was detected when the octapeptide was omitted or when a control peptide (QELLSLRR) in which the cysteine has been replaced by a serine was used for the reaction. Densitometric analyses of the autoradiograms obtained in three separate experiments are summarized

Table 1
List of the peptides assayed

β_2-AR wild-type-derived (337–344)	QELLCLRR
C341S	QELLSLRR
L339, L340 and L342	QEEECERR
	QEQQCQRR
R343 and R344	QELLCLQQ
	QELLCLEE
	QELLCLLL
E338	QQLLCLRR
	QRLLCLRR
	QLLLCLRR
Others	QELYCWRR
	QRRRCRRR
	QAAACARR

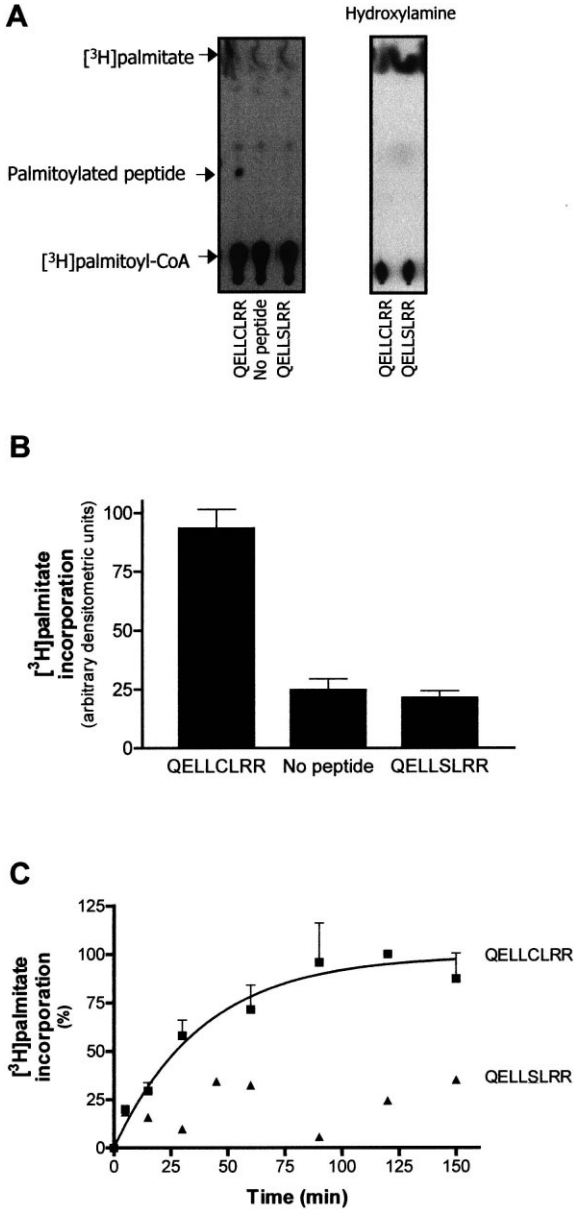

Fig. 1. *S*-Acylation of peptides derived from β_2-AR using [^{3}H]palmitoyl-CoA as acyl donor. A: *S*-Acylation of β_2-AR-derived peptides. [^{3}H]palmitate incorporation into a synthetic octapeptide corresponding to the amino acids 337–344 from the human β_2-AR primary sequence (QELLCLRR) was assessed. [^{3}H]palmitoyl-CoA (125 μCi; ~40 μM) was incubated with 1 mM of the QELLCLRR or QELLSLRR peptides or in the absence of peptide for 1 h at 37°C in 0.1 M MOPS–NaOH containing 1 mM DTT. Palmitoylated peptides were then treated or not with hydroxylamine (pH 7.4) for 1 h at 37°C. [^{3}H]palmitate incorporation was detected by autoradiography after separation of products by TLC. Data shown are representatives of three independent experiments. B: Densitometric analysis of the peptide-bound [^{3}H]palmitate. Labeling intensity was assessed by densitometric analysis of the autoradiograms. Data shown represent the means ± S.E.M. of three independent experiments. C: Kinetics of [^{3}H]palmitate incorporation. Wild-type peptide QELLCLRR and mutant peptide QELLSLRR were incubated with [^{3}H]palmitoyl-CoA for periods of time varying from 5 to 150 min. [^{3}H]palmitate incorporation is expressed as a % of the densitometric value observed following a 120 min incubation. Values shown represent the means ± S.E.M. of four independent experiments.

in Fig. 1B. The thioester nature of the linkage formed between the palmitate and the peptide was confirmed by hydroxylamine treatment, that completely removed the radioactivity associated with the peptide (Fig. 1A). Fig. 1C illustrates that the incorporation of [^{3}H]palmitate into the wild-type peptide QELLCLRR increases with time reaching a maximum after 90 min of incubation. Again, no consistent palmitate incorporation was detected using the control QELLSLRR peptide.

To determine if the thioester bond formed between QELLCLRR and [^{3}H]palmitate is spontaneously reversible at 37°C, the [^{3}H]palmitoylated peptide was incubated for 2 h with an excess of non-radioactive palmitoyl-CoA (10 mM; that corresponds to a radioisotopic dilution of more than 100×). This treatment was without effect on the extent of labeling of the peptide indicating that no rapid exchange between palmitoylated peptide and free palmitoyl-CoA occurred under these conditions. Similarly, incubation of the palmitoylated peptide with CoA (8 mM) did not reverse the reaction (data not shown).

3.2. Primary sequence determinants of S-acylation

To assess the role of the primary sequence in directing palmitoylation on a specific cysteine, the wild-type QELLCLRR peptide was divided into three distinct domains, based on the nature of the amino acids: an acidic domain (E338), a basic domain (R343, R344), and a hydrophobic domain (L339, L340 and L342). The composition of the peptide was modified to systematically change the properties of each of these domains.

To study the effect of the glutamic acid at position 338, this residue was replaced with either glutamine (QQLLCLRR), arginine (QRLLCLRR) or leucine (QLLLCLRR) to introduce a neutral, a basic or a hydrophobic character, respectively, to this position. The relative incorporation of [^{3}H]palmitate into these peptides compared to the native sequence is shown in Fig. 2A. In the three cases, substitution of E338 favored the *S*-acylation reaction indicating that the acidic domain had an inhibitory effect. Interestingly, the presence of either arginine or leucine at this position promoted a significantly greater incorporation of [^{3}H]palmitate than that observed when substituting the glutamic acid 338 for the glutamine (6- and 5-fold, respectively). This indicates not only that the acidic residue inhibits the reaction but also that the basic/hydrophobic characteristics may, on their own, have a positive effect on *S*-acylation.

To further test that basic amino acids may be positive determinants for *S*-acylation, R343 and R344 were replaced by a di-glutamine (QELLCLQQ), a di-leucine (QELLCLLL) or a di-glutamate (QELLCLEE). In all cases, the substitution of the two basic amino acids completely inhibited the *S*-acylation (Fig. 2B). This suggests that the positive charges brought by those arginines are absolutely required for the reaction.

To investigate the contribution of the hydrophobic domain to *S*-acylation, the leucine residues flanking the cysteine were first substituted with either the neutral glutamines (QEQQCQRR) or the acidic glutamates (QEEECERR). In both cases, *S*-acylation was completely inhibited (Fig. 2C) confirming that hydrophobic residues favor the reaction. To determine if the aliphatic chain of the leucines was important for this effect, L340 and L342 were changed to two aromatic residues, tyrosine and tryptophan respectively (QELYCWRR). This substitution modestly improved the palmitate incorporation arguing that the hydrophobic nature regardless of the length of the amino acid side chains is required. However, the presence of a hydrophobic environment is not essential for the reaction. Indeed, the QRRRCRRR peptide that lacks hydrophobic residues incorporated eight times more palmitate than the native sequence. However, mixed basic/hydrophobic is more favorable, as indicated by

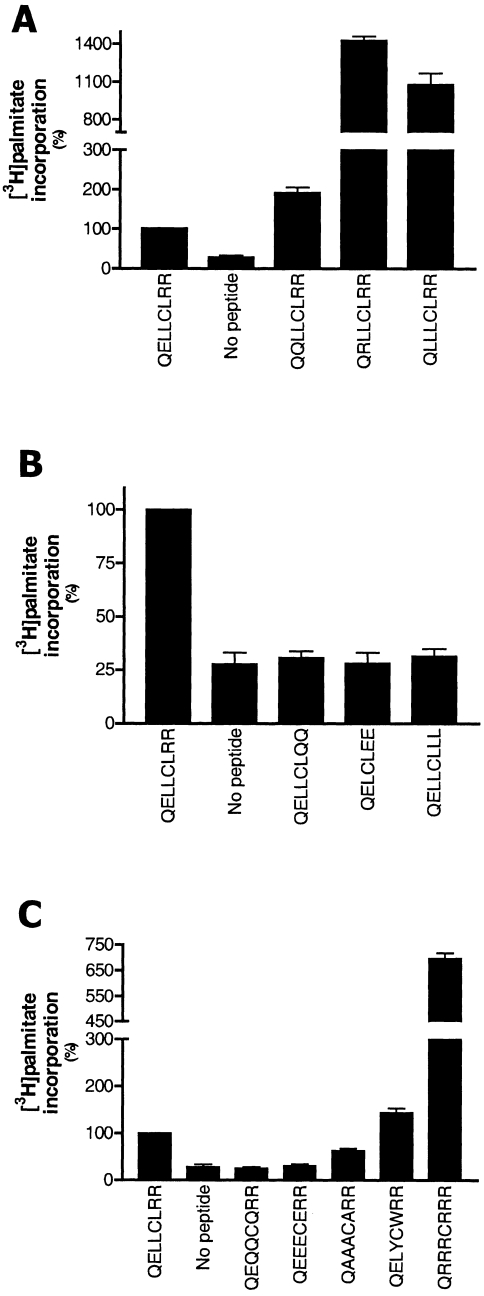

Fig. 2. Primary sequence determinant of *S*-acylation. A: Influence of the glutamic acid in position 338. B: Influence of the arginine residues at positions 343 and 344. C: Influence of the leucine residues at positions 339, 340 and 342. The indicated peptide was incubated with [^{3}H]palmitoyl-CoA for 120 min at 37°C. [^{3}H]palmitate incorporation was detected by autoradiography after separation of the products by TLC. Data are expressed as % of the densitometric value obtained for the wild-type peptide QELLCLRR, in the same experimental conditions. Data shown represent the means ± S.E.M. of three independent experiments.

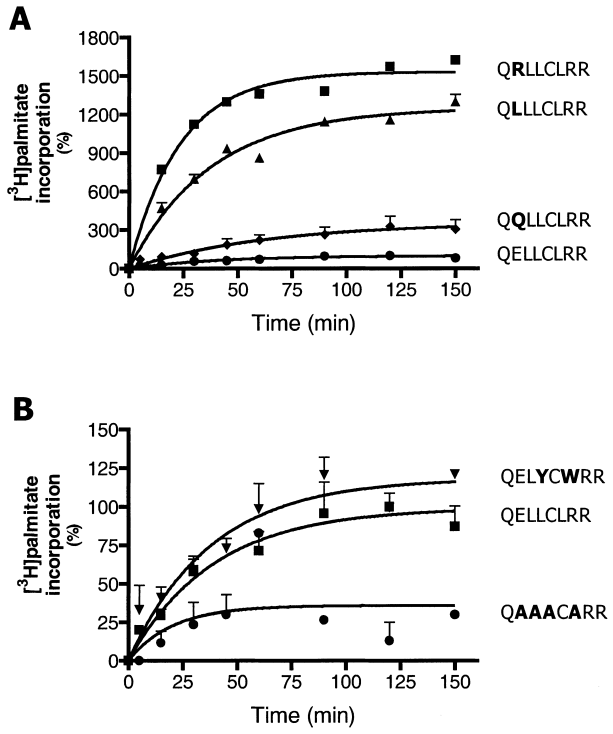

Fig. 3. Effect of the peptide primary sequence on *S*-acylation kinetics. The selected peptide and [^{3}H]palmitoyl-CoA were incubated at 37°C for times varying between 0 and 150 min. [^{3}H]palmitate incorporation was detected by autoradiography after separation of the products by TLC. Data are expressed as % of the densitometric value obtained for the wild-type peptide QELLCLRR at 120 min, pH 7.4. Data shown represent the means ± S.E.M. of four independent experiments.

the greater palmitate incorporation observed for QRLLCLRR and QLLLCLRR (11 and 14 times as compared to the native sequence, see Fig. 2A). This point is further supported by the observation that substituting L339, L340 and L342 with alanines decreases the incorporation of palmitate even when the inhibitory effect of E338 is relieved by its substitution with an alanine (QAAACARR).

3.3. *Effect of different amino acids on the kinetics of S-acylation*

The kinetics of palmitate incorporation were assessed for several peptides. In each case, the incorporation was found to be linear during the first 30 min reaching a plateau between 60 and 120 min. Fig. 3 shows that the primary sequence of the peptides affects not only the maximal level but also the initial rate of palmitate incorporation. Modification of E338 to glutamine, leucine or arginine increased the initial rate of incorporation by 2-, 12.5- and 13.5-fold, respectively (Fig. 3A). However, the replacement of E338, L339, L340 and L342 for alanines slowed the initial rate 2.5-fold below that observed for the native peptide confirming the importance of these hydrophobic residues (Fig. 3B). Replacing L340 and L342 with tyrosine and tryptophan respectively did not have a significant effect on the initial palmitate incorporation rate supporting the idea that the hydrophobic and not the aliphatic nature of the leucines is the key factor.

3.4. *Effect of pH*

It has been proposed that *S*-acylation could result from a nucleophilic attack of the sulfhydryl group of the cysteine on the thioester bond of the palmitoyl-CoA [20]. The chemical *S*-acylation would therefore be sensitive to pH as it influences the electronegativity of the cysteine sulfur. In agreement with this hypothesis, the incorporation of palmitate into the β_2AR wild-type-derived peptide was found to be influenced by pH (Fig. 4, inset). Although incorporation could be readily observed at physiological pH, it reached its maximum at pH 8.5

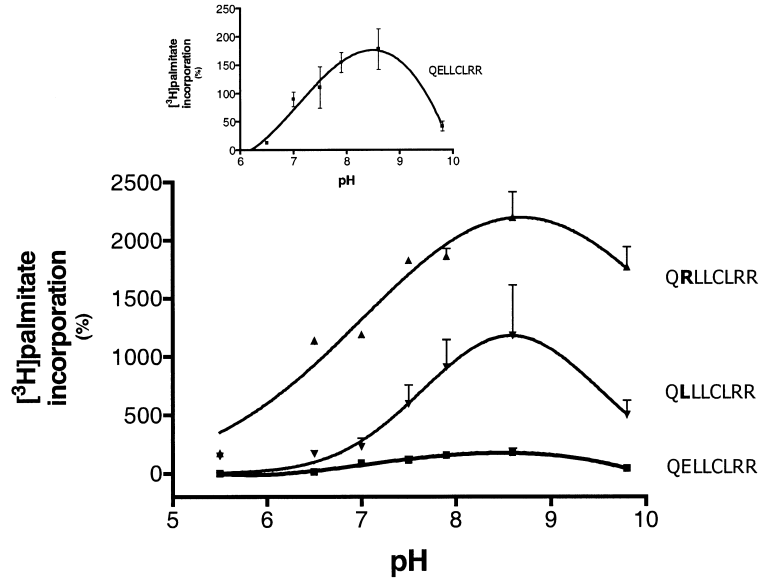

Fig. 4. Effect of pH on *S*-acylation. The indicated peptide was incubated with [^{3}H]palmitoyl-CoA in buffers of specified pH values (5.5, 6.5, 7.0, 7.4, 7.9, 8.2, 9.0) as indicated in Section 2. The incubation was carried out at 37°C for 120 min. [^{3}H]palmitate incorporation was detected by autoradiography after separation of the products by TLC. Data are expressed as % of the densitometric value obtained for the wild-type peptide QELLCLRR for 120 min at pH 7.4. Data shown represent the means ± S.E.M. of three independent experiments.

that corresponds to the pK_a of the cysteine's sulfhydryl. It was also proposed that the amino acids neighboring the cysteine would create a microenvironment that could influence its deprotonation and thus affect the optimum pH of the *S*-acylation [16]. To test the hypothesis that such a mechanism could be responsible for the increased palmitate incorporation observed upon substitution of E338 for neutral or basic residues, the influence of the pH was also studied for QRLLCLRR and QLLLCLRR. As can be seen in Fig. 4, the pH dependence of the reaction was not influenced by the substitutions. Thus, this is indicating that the presence of an acidic residue at position 338 does not modulate the extent of *S*-acylation by affecting the cysteine's pK_a.

4. Discussion

In the present study, we present the first systematic assessment of the primary sequence determinants promoting the *S*-acylation reaction. Our data clearly indicate that the presence of both hydrophobic and basic residues flanking a cysteine greatly favors its acylation, whereas acidic residues inhibit it. The observation that a high proportion of basic and/or hydrophobic residues is found proximal to the acylated cysteine in a large number of palmitoylated proteins suggests that the sequence requirements identified using the in vitro assay may also be relevant in vivo [25–33].

In vitro spontaneous *S*-acylation was previously reported for several proteins [8–21] and short peptides [10,20,22,23]. Interestingly, the reaction was found to display a significant level of selectivity [10,22,23]. Indeed, palmitoylation of glycoprotein Po was shown to occur on the same cysteine whether the protein was palmitoylated in vitro in the absence of added enzyme or in vivo [8]. Short peptides also displayed some selectivity, since only peptides containing favorable sequences could undergo *S*-acylation in vitro [10,16,20,22–24,28,34,35].

The importance of hydrophobic properties for *S*-acylation substrates was previously proposed on the basis that the presence of a myristate moiety near the cysteine is absolutely required for the palmitoylation of a peptide derived from the Yes protein kinase. In that case, it was proposed that the myristate recruits the reactive palmitoyl-CoA near the cysteine through hydrophobic interactions, favoring the spontaneous thioesterification of the cysteine [20]. Hydrophobic amino acids within protein sequences could play similar roles, promoting palmitoylation of neighboring cysteines. The presence of basic amino acids could also favor the interaction with the palmitate donor, since their positive charges could interact with the four negative charges of the CoA group. In whole cells, the salt bridges between the positively charged amino acid and the negative head groups of the plasma membrane phospholipids [16,23,36] could also contribute to the reaction by favoring the interaction between the proper peptide segment and the palmitoyl-CoA at the plasma membrane.

The important role played by flanking sequence in *S*-acylation is further upheld by the observation that the presence of acidic amino acids inhibited the reaction. Based on the mechanism proposed above, the presence of negatively charged residues could inhibit the interaction between the peptide and the palmitoyl-CoA. This may have physiological relevance on the dynamic regulation of protein palmitoylation since the presence of the negative phosphate groups on serines located three amino acids downstream from the β_2-AR palmitoylation site has previously been shown to inhibit palmitate incorporation [37].

In conclusion, our data demonstrate that in vitro *S*-acylation of small peptides displays a significant level of selectivity. Indeed, the sequence context immediately flanking the acylated cysteine determines both the rate and the extent of the reaction. The presence of basic amino acids is essential while hydrophobic and acidic residues respectively favor and inhibit the reaction. The observation that basic and hydrophobic domains are often found surrounding the palmitoylation sites of proteins may suggest that similar selectivity determinants may be at work in vivo. However, the role of these sequences in the selection of specific cysteines and the relative contribution of the autoacylation reaction to the overall palmitoylation observed in vivo will need to be further investigated.

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