Articles

Role of Acidic Amino Acids in Peptide Substrates of the β-Adrenergic Receptor Kinase and Rhodopsin Kinase†

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ABSTRACT: The β-adrenergic receptor kinase (β-ARK) phosphorylates G protein coupled receptors in an agonist-dependent manner. Since the exact sites of receptor phosphorylation by β-ARK are poorly defined, the identification of substrate amino acids that are critical to phosphorylation by the kinase are also unknown. In this study, a peptide whose sequence is present in a portion of the third intracellular loop region of the human platelet α_2-adrenergic receptor is shown to serve as a substrate for β-ARK. Removal of the negatively charged amino acids surrounding a cluster of serines in this α_2-peptide resulted in a complete loss of phosphorylation by the kinase. A family of peptides was synthesized to further study the role of acidic amino acids in peptide substrates of β-ARK. By kinetic analyses of the phosphorylation reactions, β-ARK exhibited a marked preference for negatively charged amino acids localized to the NH_2-terminal side of a serine or threonine residue. While there were no significant differences between glutamic and aspartic acid residues, serine-containing peptides were 4-fold better substrates than threonine. Comparing a variety of kinases, only rhodopsin kinase and casein kinase II exhibited significant phosphorylation of the acidic peptides. Unlike β-ARK, RK preferred acid residues localized to the carboxyl-terminal side of the serine. A feature common to β-ARK and RK was a much greater K_m for peptide substrates as compared to that for intact receptor substrates.

The β-adrenergic receptor is a member of a growing family of recently cloned G protein coupled receptors (O'Dowd et al., 1989). These proteins have a number of structural and functional similarities to the visual pigment rhodopsin (Lefkowitz & Caron, 1988). Both Rho[1] and β-AR are membrane-associated glycoproteins with seven intramembranous domains connected by both intra- and extracellular loops. The amino-terminal portion of these molecules, which contains the sites of N-linked glycosylation, is oriented toward the extracellular space, while the carboxyl-terminal portion of the receptor is directed toward the cytosol of the cell. Upon adsorption of a photon of light by Rho or agonist occupancy of the β-AR, the receptors stimulate intracellular second messenger pathways via the interaction with specific G proteins. The ability to activate the G protein is regulated, in part, by phosphorylation of the receptor. Recent work has demonstrated that both Rho and β-AR undergo phosphorylation in a signal-dependent manner by a novel class of protein kinases. Rhodopsin kinase phosphorylates photobleached rhodopsin, while the agonist-occupied form of the β_2-AR serves as a substrate for the β-adrenergic receptor kinase. The substrate specificity of β-ARK is not limited to the β-AR as additional receptor substrates of β-ARK include the human platelet α_2-adrenergic receptor (Benovic et al., 1987a), the chick heart muscarinic receptor (Kwatra et al., 1989), and metarhodopsin II, which is a poor substrate for the enzyme (Benovic et al., 1986a).

The physiological significance of these signal-dependent phosphorylation events is best understood in the visual transduction pathway. When photoactivated, rhodopsin undergoes a series of well-characterized conformational changes. One of the photointermediates (metarhodopsin II) interacts with several cytosolic proteins including transducin, a G protein present in the retina. This interaction promotes the exchange of GDP for GTP on the α subunit (T_α) of transducin (Fung & Stryer, 1980) and activates the second messenger system, cGMP phosphodiesterase (Hurley & Stryer, 1982), present in the retinal cell. However, an efficient mechanism for quenching or terminating the visual signal is required since the long half-life of metarhodopsin II would result in the

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[1] Abbreviations: α_2AR, α_2-adrenergic receptor; β-AR, β-adrenergic receptor; β_2-AR, β_2-adrenergic receptor; β-ARK, β-adrenergic receptor kinase; BSA, bovine serum albumin; CK-II, casein kinase II; EDTA, ethylenediaminetetraacetic acid; fMOC, *N*-(9-fluorenylmethoxycarbonyl); G_s, stimulatory guanine nucleotide binding protein; HPLC, high-performance liquid chromatography; NH_2 terminal, amino terminal; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKI, heat-stable cAMP-dependent protein kinase inhibitor peptide; Rho, rhodopsin; RK, rhodopsin kinase; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane. IUPAC single-letter abbreviations for amino acids are used throughout.

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continued activation of the G protein for several minutes following light adsorption (Kühn & Wilden, 1987). This quenching mechanism involves the interaction of metarhodopsin II with two cytosolic proteins, rhodopsin kinase and arrestin. Phosphorylation of light-activated rhodopsin by RK is the key step in the signal-termination reaction. In vitro, phosphorylated rhodopsin is less effective in stimulating the phosphodiesterase (Sitaramayya & Liebman, 1983a,b; Aton & Litman, 1984; Wilden et al., 1986). Following the phosphorylation by RK, the photoreceptor now binds arrestin, which sterically hinders transducin activation (Kühn et al., 1985). The combined actions of RK and arrestin turn off the photoactivation pathway long before the decay of metarhodopsin II to its inactive intermediates.

An analogous mechanism exists in the β-adrenergic receptor/G_s system. The β_2-AR undergoes agonist-dependent phosphorylation by the enzyme β-ARK to a maximal stoichiometry of ≈8 mol PO_4/mole of receptor (Benovic et al., 1987b). As assessed with a reconstituted system, β-ARK-mediated phosphorylation of the β_2-AR impairs coupling to the G protein. This inhibition is potentiated by the addition of purified retinal arrestin (Benovic et al., 1987c). Recently, β-arrestin, a protein with increased specificity for the β-AR as compared to Rho, was cloned by virtue of its high degree of homology with retinal arrestin (Lohse et al., 1990a). When a reconstituted system is used, the combined actions of β-ARK and β-arrestin markedly decrease the ability of the β_2-AR to activate G_s.

In contrast to the visual system, little is known concerning the sites of β_2-AR phosphorylation by β-ARK. Rhodopsin phosphorylation by RK occurs on a cluster of serines and threonines in the carboxyl-terminal portion of the molecule (Thompson & Findlay, 1984) and the intervening loop between transmembrane helices V and VI (Applebury & Hargrave, 1986). When reconstituted β_2-AR and purified β-ARK are used, proteolysis experiments suggest that the sites of phosphorylation are localized to the carboxyl-terminal portion of the receptor (Dohlman et al., 1987). Studies with synthetic peptides demonstrate that two acidic peptides with amino acid sequences found in the carboxyl-terminal portion of the β_2-AR serve as substrates for β-ARK (Benovic et al., 1990). In the present study, we have used synthetic peptides to further define the substrate determinants for β-ARK and compare these results to the related visual system enzyme RK. These data provide information concerning substrate specificity and identify potential β-ARK phosphorylation sites present in a number of G protein coupled receptors.

Experimental Procedures

Materials. Chemicals were from sources previously described (Benovic et al., 1984, 1987b). Peptides were synthesized on an Applied Biosystems 430A synthesizer using fMOC chemistry. Prior to use, the peptides were purified by high-performance reverse-phase chromatography on a C-18 column with a 0–50% acetronitrile gradient in 0.1% trifluoroacetic acid. BSA, heparin, isoproterenol, and PKI (rabbit muscle) were obtained from Sigma Chemical Company. P-81 paper was from Whatman. AG X-18 columns were purchased from Bio-Rad, and reverse-phase columns were purchased from Vydac. [γ-^{32}P]ATP was from New England Nuclear.

Purification of the β-Adrenergic Receptor Kinase and Rhodopsin Kinase. β-ARK was purified from bovine cerebral cortex as previously described (Benovic et al., 1987b, 1990). β-ARK activity was precipitated from a high-speed supernatant fraction with ammonium sulfate and the resuspended material chromatographed on an Ultragel AcA34 column. The peak kinase activity was then applied sequentially to DEAE Sephacel and CM Fractogel columns. The β-ARK activity eluted from the DEAE column at a much lower ionic strength than did the bulk of the contaminating kinases. The β-ARK preparation after the CM Fractogel step is approximately 70% pure. The addition of 0.02% Triton X-100 to the buffers after the AcA34 step stabilized the kinase activity for months when stored at 4 °C. The recent cloning of the cDNA for bovine β-ARK and the evidence of a multigene family (Benovic et al., 1989a) raises the possibility that our purified bovine brain β-ARK preparation contains multiple β-ARK-related enzymes. In fact, we have recently cloned a related kinase from a bovine cDNA library (J. L. Benovic, manuscript in preparation). While we cannot categorically rule out the possibility of an additional kinase in our β-ARK preparation, several lines of evidence suggest that the bovine cerebral cortical preparation does not contain the β-ARK-related species that we have cloned. First, mRNA levels for the β-ARK-related enzyme were significantly lower (≈10-fold) than for β-ARK itself in bovine cerebral cortex. Second, the elution profile of our purified bovine brain β-ARK on a Mono S column was identical with the cloned β-ARK expressed in COS-7 cells and significantly different than the β-ARK-related enzyme. Finally, our purified β-ARK preparation and the cloned β-ARK were equally sensitive to inhibition by heparin, while the β-ARK-related enzyme was ≈100-fold less sensitive to heparin.

Rhodopsin kinase was prepared as described by Palczewski et al. (1988). This procedure made use of the property that rhodopsin kinase binds to photobleached rod outer segment membranes while other retinal kinases remain in the cytosol. The rhodopsin kinase was then extracted from the ROS membranes with 60 mM KCl. Following adjustment of the pH and dilution of the extract, the kinase was further purified with a DEAE–cellulose column. Pooled kinase activity from the DEAE step was directly applied to a hydroxyapatite column and eluted with a high-salt buffer. The specific activity was 500 to 850 nmol of PO_4 transferred/(min·mg of kinase).

The catalytic subunit of cAMP-dependent protein kinase was prepared from bovine cardiac muscle by the method of Reimann and Beham (1983) using DEAE-cellulose and hydroxyapatite chromatography. Protein kinase C and casein kinase II were generous gifts of Dr. Perry Blackshear (Duke University Medical Center, Durham, NC) and Dr. Edwin Krebs (University of Washington, Seattle, WA).

Phosphorylation of Synthetic Peptides. A stock solution of purified synthetic peptides was prepared and the pH adjusted to 7.4 with a small amount of Tris-base. The peptides were incubated with various amounts of β-ARK (0–20 ng) in a buffer containing 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 7.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM [γ-^{32}P] ATP (250–500 cpm/pmol), 10 μM PKI, and 0.5 mg/mL BSA in a final volume of 25 μL at 30 °C. The reaction was stopped by transferring the entire reaction mixture to a 2 × 2 cm square of P-81 paper followed by five washes in 75 mM phosphoric acid (10 mL/square of paper) as previously described (Cook et al., 1982). β-ARK activity was defined as the difference in phosphate incorporation in the presence and absence of peptide. Comparable results were obtained if kinase activity was defined in the presence and absence of β-ARK. For kinetic experiments, the purity of the kinase preparation was determined (≈70%) and the values for the V_{max} were adjusted accordingly. A nonlinear regression program (Enzfitter, Elsevier-Biosoft, Cambridge, U.K.) was used to

estimate the kinetic parameters.

A second method for the identification of phosphopeptides involved sequential ion-exchange and reverse-phase chromatography. A phosphorylation reaction was stopped with 0.5 mL of 30% acetic acid. The entire sample was then applied to an AG 1-X8 column (2-mL bed volume) equilibrated in 30% acetic acid. The peptide was eluted with 2 mL of 30% acetic acid while the bulk of the ATP remained bound to the column. The peptide was then dried under reduced pressure (Speed-Vac) and rehydrated in 100 μL of 0.1% TFA. The sample was applied to a C-18 reverse-phase HPLC column and eluted with a 0–25% acetonitrile gradient monitored for both absorbance at 214 nm and ^{32}P radioactivity. The phosphopeptide was again dried and prepared for SDS–urea polyacrylamide gel electrophoresis or phosphoamino acid analysis as described below.

Phosphorylation of Bovine Rhodopsin. Rod outer segments were prepared according to slight modifications in the procedures of Wilden and Kühn (1982) and were urea treated to remove endogenous kinase activity as described by Shichi and Somers (1978). The rod outer segments contained approximately 95% rhodopsin with negligible kinase activity. Phosphorylation of the rod outer segments by β-ARK was performed as described previously (Benovic et al., 1987b).

Phosphoamino Acid Analysis. Following hydrolysis in 6 N HCl at 105 °C for 1 h, [^{32}P]phosphoamino acids were resolved by high-voltage electrophoresis on a cellulose plate and subsequently localized with autoradiography (Cooper et al., 1983).

Amino Acid Sequencing. N-Terminal amino acid sequencing was performed by Dr. Richard Randall (Howard Hughes Medical Institute, Duke University, Durham, NC) on an Applied Biosystems 470A sequenator (Speiss et al., 1979; Hewik et al., 1981).

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) with 10 or 15% slab gels. SDS–urea polyacrylamide gel electrophoresis was carried out with 8 M urea and 9% acrylamide (Swank & Munkres, 1971).

Results

Previous work has demonstrated that acidic peptides with sequences present in the carboxyl-terminal region of the β_2-AR were substrates for β-ARK (Benovic et al., 1990). To further examine the substrate characteristics of peptides, a synthetic peptide whose sequence is based on a portion of the human α_2-adrenergic receptor third intracellular loop (LEESSSSDHAERPPG) was studied (Kobilka et al., 1987). Figure 1a demonstrates the β-ARK-mediated phosphorylation of this peptide. When analyzed by electrophoresis in a SDS–urea system, the peptide exhibited anomalous migration with an apparent mobility of 7000 Da. As shown in Figure 1b, the isosteric substitution of the acidic residues (E → Q) and (D → N) surrounding the serine cluster resulted in the complete loss of phosphorylation (LQQSSSSNHAERPPG). The autoradiogram has been overexposed in an attempt to detect phosphorylation of the substituted peptide, which was negligible. The identity of the phosphopeptide was confirmed by initially purifying the peptide with sequential ion-exchange and reverse-phase HPLC followed by gas-phase amino acid sequencing. In addition, the phosphopeptide isolated by the chromatographic procedure comigrated in the SDS–urea gel system with the observed ^{32}P-labeled peptide and yielded [^{32}P]phosphoserine upon hydrolysis (data not shown). Moreover, phosphorylation of the α_2-AR third-loop peptide (Figure 1b) was inhibited by heparin, a potent inhibitor of

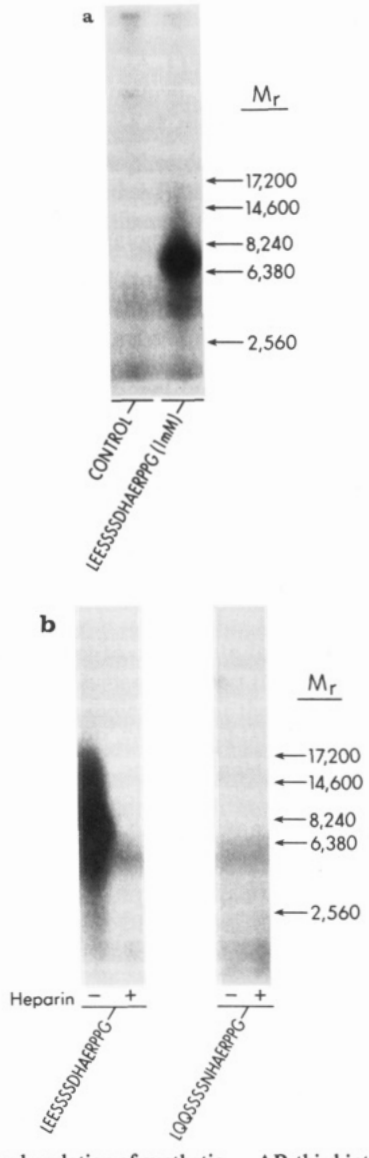

FIGURE 1: Phosphorylation of synthetic α_2-AR third intracellular loop peptides. (a) Phosphorylation of the native α_2-AR third intracellular loop peptide. β-ARK was incubated in the presence or absence of a synthetic peptide based on a sequence present in the platelet α_2-AR third intracellular loop (LEESSSSDHAERPPG). The final concentration of peptide was 1 mM. After 2 h at 30 °C, the reaction was stopped by the addition of SDS–urea stop solution followed by electrophoresis employing a 9% acrylamide/8 M urea gel system. The phosphopeptide was visualized by autoradiography at −70 °C. (b) Effect of removal of charged residues in the region of the serine cluster and heparin sensitivity. The platelet α_2-AR third intracellular loop (LEESSSSDHAERPPG) peptide and a modification in which the acidic amino acids surrounding the serine cluster were changed to the uncharged counterparts (LQQSSSSNHAERPPG) were incubated with β-ARK as described in the text. The final concentration of peptide in each case was 1 mM. After 2 h at 30 °C, the peptides were prepared for electrophoresis as described above. Each incubation was performed in the absence (−) or presence (+) of 100 nM heparin.

β-ARK (Benovic et al., 1989b).

The results obtained with the α_2-AR peptides were consistent with the hypothesis that acidic amino acids are required for a peptide to serve as a substrate of β-ARK. However, since the α_2-AR and β_2-AR peptides contain multiple serine and/or threonine residues and the exact sites of phosphorylation were not identified, any spatial relationship between the phosphate acceptor group and acidic residues could not be assessed. An alternative approach involved the synthesis of a family of peptides in which the structure was systematically varied. When the ability of the peptides to serve as β-ARK substrates

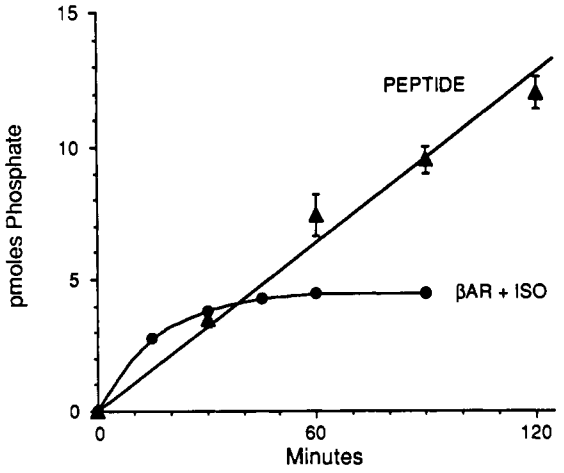

FIGURE 2: Time course of phosphorylation of the β_2-AR and the parent peptide (RRREEEEESAAA) by β-ARK. Reconstituted agonist-occupied [50 μM (−) isoproterenol] hamster lung β_2-adrenergic receptor (≈0.5 pmol) (●) and the synthetic peptide RRREEEEESAAA (1.25 mM) (▲) were incubated with β-ARK as described in the text. At various times, 25-μL aliquots of the reaction mixture were stopped by the addition of 50 μL of SDS sample buffer (β_2-AR) or spotting on P-81 paper (peptide). The receptor sample was subjected to electrophoresis on a 10% acrylamide gel system. The receptor bands were localized with autoradiography and then excised and counted to determine the picomoles of phosphate incorporated. The peptide was washed in phosphoric acid as described in the text, and results were expressed as picomoles of phosphate incorporated into the peptide. The results for the receptor phosphorylation are presented as a single representative experiment, while the peptide data are expressed as the mean ±SD with $n = 3$.

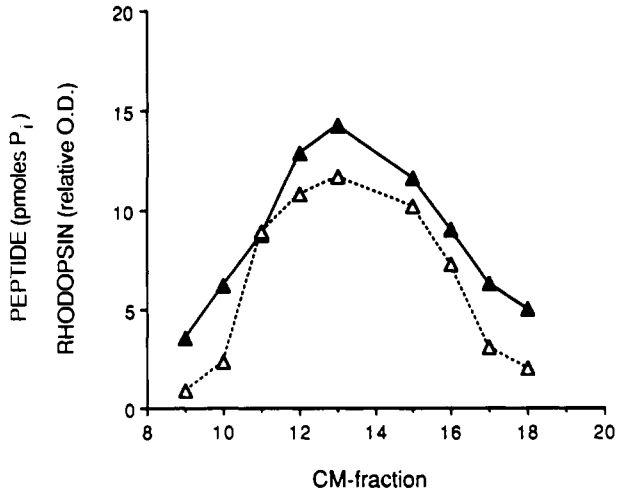

FIGURE 3: CM–Fractogel profile for β_2-AR and the synthetic peptide RRREEEEESAAA. Light-activated urea-treated rod outer segments (≈5 μg of rhodopsin) (△) or the peptide RRREEEEESAAA (5 mM final) (▲) were incubated with 5-μL aliquots of each fraction obtained from the CM–Fractogel step of the β-ARK purification. After 20 min the rhodopsin phosphorylation reaction was stopped by the addition of SDS sample buffer, and the samples were loaded on a 9% acrylamide gel. An autoradiogram was prepared, and rhodopsin phosphorylation was quantitated by scanning densitometry and expressed as relative optical density. The peptide phosphorylation was expressed as picomoles of phosphate incorporated following a 100-min incubation as described in the text.

(defined by their kinetic parameters) was compared, the role that acidic amino acids play in defining the substrate specificity of β-ARK could be more systematically addressed. Two characteristics of this family of peptides facilitated these studies. First, the amino-terminal arginine groups provided an easy assay for the phosphorylation reaction. The peptides are bound to ion-exchange paper and washed free of excess [^{32}P]ATP, and ^{32}P incorporation is quantitated by liquid-scintillation techniques. This permits the analysis of the large number of reactions necessary to study the kinetics of phosphorylation. Second, the model peptides contain a single serine or threonine that serves as the phosphorylation site. To begin, an acid-rich parent peptide was synthesized (RRREEEEESAAA) that contained a cluster of glutamates similar to the α_2-AR peptide. Figure 2 compares the time course of β-ARK-catalyzed phosphorylation of agonist-occupied β_2-AR and the parent peptide. In contrast to peptide phosphorylation, which was linear for at least 2 h, receptor phosphorylation exhibited a plateau as maximal stoichiometry for β_2-AR phosphorylation was approached (6–8 mol PO_4/mol β-AR).

Since the β-ARK preparation used in these studies was 70% pure, the following experiment was performed to substantiate that β-ARK was the enzyme responsible for the phosphorylation of the peptide substrates. Figure 3 compares the phosphorylation profile of the parent peptide to that of β-ARK activity (defined as the ability to phosphorylate bleached rhodopsin) by using various fractions obtained from the elution of the CM Fractogel column. The profile of β-ARK activity parallels the kinase activity responsible for phosphorylating the synthetic peptide. Additionally, the phosphorylation of both rhodopsin and the peptide was inhibited by heparin. These observations confirm that β-ARK was the enzyme responsible for the phosphorylation of the peptide.

Table I presents the kinetic data for the parent and several related synthetic peptides. The parent peptide has a V_{max} of

Table I: β-ARK Phosphorylation of Acid-Rich Synthetic Peptides[a]

substrate	K_m (mM)	V_{max} [nmol P_i/(min·mg)]	V_{max}/K_m
RRREEEEESAAA	1.1 ± 0.2	28.9 ± 1.3	26
RRRDDDDDSAAA	1.3 ± 0.2	24.0 ± 1.5	18
RRRAAAAASEEE	nd[b]	nd	
RRREEEEETAAA	0.9 ± 0.2	6.5 ± 0.4	7
RRREEEEESEEE		9.4 ± 3.3	

[a] Various peptides (0–5 mM) were incubated with β-ARK for 100 min at 30 °C as described in the text. The kinetic parameters were determined by a nonlinear regression program (Enzfitter) and expressed as the mean ±SE with $n = 3$–6. A K_m could not be reliably estimated for RRREEEEESEEE as discussed in the text. The V_{max} for this peptide was based on values obtained at peptide concentrations ≤0.5 mM. [b] nd = no detectable phosphorylation.

29 nmol/(min·mg), which is comparable to that seen with the intact receptor (Benovic et al., 1987b, 1990). However, the K_m is 1.1 mM, a value almost four orders of magnitude greater than that observed with the receptor. This difference in K_ms has been observed with all reported peptide substrates of both β-ARK (Benovic et al., 1990) and RK (Palczewski et al., 1988, 1989; Kelleher & Johnson, 1990). Replacement of glutamate residues (E) with aspartate (D) (RRRDDDDDSAAA) yielded similar kinetic values (V_{max} = 24 nmol/(min·mg) and K_m = 1.3 mM). When the cluster of glutamic acid residues was moved to the carboxyl-terminal side of the serine (RRRAAAAASEEE), there was no detectable phosphorylation of the peptide. The phosphorylation of the peptide RRREEEEESEEE was biphasic. At low concentrations of peptide (≤0.5 mM), the calculated V_{max} was 9.4 nmol/(min·mg), while at higher concentrations of peptide, inhibition of phosphorylation was observed. Since saturation of the enzyme was not achieved, the K_m could not be determined and the V_{max} may, in fact, be an underestimate. The inhibition at high peptide concentration was not observed with rhodopsin kinase (see below), which argues against aggregation or nonspecific interactions of the peptide as an explanation for the biphasic response. Exchanging the serine with threonine

Table II: β-ARK Phosphorylation of Synthetic Peptides Containing a Single Glutamic Acidic Residue[a]

substrate	K_m (mM)	V_{max} [nmol P_i/(min·mg)]	V_{max}/K_m
RRRAAAAESAAA	3.5 ± 1.4	6.4 ± 1.1	2
RRRAAAEASAAA	1.5 ± 0.6	22.5 ± 3.5	15
RRRAAEAASAAA	4.8 ± 1.4	21.1 ± 3.1	4
RRRAEAAASAAA		12.3 ± 7.6	
RRREAAAASAAA	4.3 ± 3.1	9.3 ± 3.2	2

[a] Experimental conditions were exactly as described in Table I. The results are presented as the mean ±SE with $n = 3$. A K_m could not be reported for the peptide RRRAEAAASAAA due to limited solubility as reported in the text.

Table III: Activity of Various Kinases Toward Two Acid-Rich Peptide Substrates[a]

kinase	protein[b]	RRREEEEESAAA	RRRAAAAASEEE
β-ARK	32.8	9.8 (0.3)[c]	nd[d]
RK	1200	5.1 (0.004)	18 (0.015)
PKA	1800	nd	nd
PKC	24.5	nd	nd
CK-II	339	12.5 (0.04)	240 (0.71)

[a] Conditions for PKA and PKC phosphorylation were as described by Bouvier et al. (1987), and CK-II activity was assayed as outlined by Kuenzel and Krebs (1985). Results were expressed as picomoles of phosphate incorporated into substrate per 40 min. The final peptide concentration was 1 mM. Each assay contained 10 ng of CK-II, PKA, or PKC while β-ARK and RK were present at 25 ng/assay. [b] For β-ARK and RK, light-exposed ROS served as a protein substrate. PKA and PKC employed histone as a substrate; CK-II was assayed with casein. [c] Ratio of the rate of phosphorylation of the protein substrate to the peptide substrating during 40 min. [d] nd = not detectable.

(RRREEEEETAAA) resulted in a 4-fold decrease in the V_{max} [6 nmol/(min·mg)] with no appreciable change in the K_m (0.9 mM). These results clearly demonstrate that peptide substrates of β-ARK require acidic residues to be localized on the NH_2-terminal side of a phosphate acceptor group with serine as the preferred residue to be phosphorylated.

To further define the role of acidic residues in substrates of β-ARK, a family of peptides was synthesized in which a single glutamate was progressively moved further from the serine residue. The data for this family of five peptides are found in Table II. Of this group, the peptide RRRAAAEASAAA proved to be the best substrate with kinetic parameters [V_{max} = 22 nmol/(min·mg) and K_m = 1.5 mM] similar to the parent peptide RRREEEEESAAA. When the glutamate was adjacent to the serine residue (RRRAAAAESAAA), there was a 7-fold decrease in the overall catalytic efficiency (V_{max}/K_m ratio) due to an increase in the K_m (3.5 mM) and a fall in the V_{max} [6 nmol/(min·mg)]. The ability of the peptide to serve as a substrate for β-ARK grows progressively worse as one moves the glutamic acid 3–5 residues from the serine.

A variety of kinases were tested for their ability to phosphorylate two acidic peptides, each with the cluster of glutamic acids on either side of the phosphate acceptor group. As shown in Table III, neither PKA nor PKC utilized the peptides as substrates. Unlike β-ARK, which required acidic amino acids on the NH_2-terminal side of a serine, RK and CK-II phosphorylated both peptides. On the basis of the kinetic analysis of phosphorylation reactions with three acid-rich peptides, RK demonstrated a preference for acidic residues localized to the carboxy-terminal side of the phosphate acceptor group (Table IV).

Table IV: Rhodopsin Kinase Phosphorylation of Acid-Rich Peptides[a]

substrate	K_m (mM)	V_{max} [nmol P_i/(min·mg)]	V_{max}/K_m
RRREEEEESAAA	2.0 ± 0.3	2.1 ± 0.9	1.1
RRRAAAAASEEE	1.8 ± 0.2	8.0 ± 1.0	4.4
RRREEEEESEEE	1.5 ± 0.3	4.8 ± 1.8	3.2

[a] The kinetic parameters were determined as described under Materials and Methods. A total of 2.3 μg of rhodopsin kinase was incubated with various concentrations of the selected peptides in a final volume of 600 μL. The reaction mix contained 10 mM bis-Tris-propane, 2 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (350 cpm/pmol), and 1 mM DTT, pH 7.4, at 30 °C. After 40 min, aliquots were applied to P-81 paper and washed with phosphoric acid (75 mM) as described in the text.

Discussion

The regulation of receptor function frequently involves desensitization, a process in which a cell exposed to a hormone becomes less responsive to subsequent stimulation. Desensitization occurs in a wide variety of signaling pathways (Sibley et al., 1987). In the β-adrenergic receptor–adenylyl cyclase system, both nonselective (heterologous) and agonist-specific (homologous) forms of desensitization involve receptor phosphorylation (Benovic et al., 1988; Strader et al., 1989; Hausdorff et al., 1990). Thus, β-ARK, by virtue of its agonist dependence for receptor phosphorylation, has been implicated in the mechanism of rapid homologous desensitization.

Three lines of evidence support this role of β-ARK. First, Green and Clark (1981) clearly demonstrated that the homologous form of desensitization occurred in mutant S49 cell lines deficient in cAMP-dependent protein kinase activity (*kin*$^-$) or G_s protein α subunit (*cyc*$^-$). Therefore, the cAMP-dependent protein kinase did not appear to be involved in homologous desensitization. Subsequently, Strasser et al. (1986) reported that agonist-induced receptor phosphorylation accompanied desensitization in the same cell lines. A crude cytosolic fraction from the *kin*$^-$ cell line contained an enzyme that phosphorylated the β_2-AR in an agonist-dependent manner (Benovic et al., 1986b). In this way, the agonist-dependent but cyclic nucleotide-independent kinase, β-ARK, was identified (Benovic et al., 1987b). Secondly, Lohse et al. (1989, 1990b) used a permeabilized cell system to demonstrate that β-ARK inhibition by heparin blocked both receptor phosphorylation and homologous desensitization. While the exact residues within the β-adrenergic receptor modified by β-ARK are unknown, Dohlman et al. (1987) localized the phosphorylation sites to the carboxyl-terminal region of the receptor. Cells that express a mutant receptor lacking the serine/threonine-rich tail of the β_2-AR fail to undergo agonist-dependent phosphorylation, and agonist-specific desensitization is delayed (Bouvier et al., 1988). Homologous desensitization is also delayed in cells that express a mutant β_2-AR in which the serine and threonine residues present in the carboxyl-terminal portion of the receptor were replaced with alanine and glycine (Bouvier et al., 1988; Hausdorff et al., 1989). Mechanisms involved in delayed desensitization appear normal and may be due to receptor sequestration, which is not altered in these cell lines (Strader et al., 1987; Bouvier et al., 1988). Thus, the available data strongly suggest that agonist-dependent phosphorylation, via β-ARK, is directly involved in the early stages of homologous desensitization.

Despite the potential physiologic importance of this family of kinases, little is known concerning substrate specificity of β-ARK or the related enzyme RK. Previously, synthetic peptides based on sequences from known phosphorylation sites found in native protein substrates have proved useful in defining the determinants critical for kinase recognition and phosphorylation. As an example, modifications of a peptide based on the cAMP-dependent protein kinase phosphorylation site in pyruvate kinase demonstrated the importance of basic amino acids for kinase activity (Kemp et al., 1977). A second

approach involves the synthesis of peptides that resemble intact protein phosphorylation sites insofar as they contain the residues important for substrate recognition. The use of peptides containing clusters of acidic amino acids on the carboxyl-terminal side of a serine/threonine allowed investigators to define the substrate specificity for casein kinase II (Meggio et al., 1984; Marin et al., 1986; Kuenzel et al., 1987).

In the present study, we used a combination of both approaches to examine the specificity of β-ARK. Previous work suggested that the carboxyl-terminal region of the hamster β_2-AR contained the putative β-ARK phosphorylation sites (Dohlman et al., 1987). In support of this finding was the observation that out of 11 peptides tested, encompassing most of the intracellular and extracellular domains of the β_2-AR, only two peptides were phosphorylated by β-ARK (Benovic et al., 1990). Both of these peptides had amino acid sequences based on regions in the carboxy-terminal region of the β_2-AR. An additional feature common to both substrate peptides was the presence of acidic amino acids in close proximity to serine and threonine residues. In this study, a peptide with similar properties, present in the third intracellular loop of the human platelet α_2-AR (LEESSSSDHAERPPG), was identified. When studied, this peptide was also found to be a substrate for β-ARK. A modification in which the acidic amino acids surrounding the serine cluster were changed to their uncharged counterparts (LQQSSSSNHAERPPG) demonstrated that negatively charged residues are required for a peptide to serve as a substrate of β-ARK.

In order to delineate the acidic amino acid requirement for the kinase, a parent peptide containing a cluster of five glutamates (RRREEEEESAAA) and a family of related peptides were characterized. When these peptides were used, a requirement for NH_2-terminal acidic residues relative to the phosphate acceptor group was established. While the preference for NH_2-terminal acidic amino acids was clear, the exact spatial arrangement of charged residues was less well-defined. On the basis of a series of homologous peptides in which the position of a single glutamic acid was varied, a 7-fold difference in catalytic efficiency (V_{max}/K_m ratio) was found.

Of note was the dramatic difference in the K_m of native protein substrates when compared to the peptide substrates. Both β-ARK and rhodopsin kinase were found to have 100–10000-fold differences in the K_m between intact receptor and peptides as substrates (Palczewski et al., 1988; Kelleher & Johnson, 1990; Benovic et al., 1990). Both kinases are thought to interact with multiple domains of their respective receptor substrates. In support of this was the observation that peptides from a variety of intracellular regions of the β_2-AR specifically inhibited β-ARK phosphorylation of the intact β_2-AR but not the phosphorylation of peptide substrates of β-ARK (Benovic et al., 1990). Additionally, three groups have previously reported that RK bound to bleached rhodopsin was still capable of phosphorylating peptide substrates (Fowels et al., 1988; Palczewski et al., 1988; Kelleher & Johnson, 1990). These observations are consistent with the hypothesis that the kinase binds intact receptor substrates at sites distinct from the actual site of phosphorylation. In light of these data, it is reasonable to suggest that the overall affinity of the kinase for its substrate, and hence the K_m, is a function of these multiple sites of interaction. Since the peptide substrates lack most of the determinants required to stabilize binding to the kinase, they exhibit a dramatically increased K_m with a V_{max} comparable to that observed with receptor substrates.

Despite the high K_m of these peptide substrates, both β-ARK and RK discriminate in their ability to phosphorylate peptides. Therefore, information as to the substrate specificity of these kinases is available through the study of peptide phosphorylation. Rhodopsin kinase specifically phosphorylates only those peptides with sequences based on known phosphorylation sites present in intact opsin, while peptides from other intracellular regions of rhodopsin did not serve as substrates (Palczewski et al., 1989; Kelleher & Johnson, 1990). In addition, RK phosphorylated a number of acid-rich peptides from the carboxy termini of other visual pigments and the β_2-AR as well as a peptide from the third intracellular loop of the M_1 muscarinic receptor (Palczewski et al., 1989). Previously, we examined a number of peptides with amino acid sequences found in the β_2-AR (Benovic et al., 1990). It is our contention that peptides which served as substrates for β-ARK contain the putative phosphorylation sites present in the intact β_2-AR.

The present report extends these original observations to the α_2-AR. Our data demonstrate that a synthetic peptide with a sequence found in the third intracellular loop of the α_2-AR is a substrate for β-ARK. We suggest that the previously reported phosphorylation of the α_2-AR occurs in this region of the intact receptor (1987a). Additionally, we report the importance of negatively charged residues in substrates of β-ARK and the related enzyme RK. When the spatial relationship of the charged residues to the site of phosphorylation is compared, subtle differences exist between β-ARK and RK. β-ARK required acidic amino acids on the NH_2-terminal side of the phosphorylated group. Peptides with glutamates alone on the carboxyl-terminal side of a serine were not β-ARK substrates. A peptide with glutamates on both sides of the serine residue (RRREEEEESEEE) was a poor substrate at low peptide concentrations and was not appreciably phosphorylated at higher concentrations. While not proven, this suggested that β-ARK may be inhibited by negatively charged residues on the carboxyl-terminal side of the phosphate acceptor group. In contrast, RK phosphorylated peptides with glutamates on either side of the serine residue. On the basis of the kinetic analyses, RK exhibited a preference for acidic residues localized to the carboxyl- as compared to the amino-terminal side of the phosphate acceptor group.

The lack of a rigorously defined consensus amino acid sequence may well reflect an important property of the kinase. Both β-AR and rhodopsin kinase phosphorylate multiple sites in relatively discrete regions of their intact substrates. We hypothesize that the multiple sites of interaction between the kinase and receptor serve to properly align the two proteins. The acidic regions of the substrate serve to identify specific serine/threonine residues in different domains of the receptor (i.e., the carboxy tail of Rho and β_2-AR and the third intracellular loops of Rho; α_2-AR, and muscarinic receptors) as sites of phosphorylation. The overall specificity of this family of kinases stems from their ability to interact with multiple regions of their intact receptor substrates. While the data suggest that acidic amino acids near the site of phosphorylation are important, these residues only partially contribute to the interaction of the kinase and substrate. It follows that an acidic nonreceptor protein could serve as a substrate, albeit poor relative to the β_2-AR or Rho. This appears to be the case as both β-ARK (Benovic et al., 1990) and RK (Palczewski et al., 1989) can phosphorylate casein to a much lesser extent than their receptor substrates.

On the basis of the requirement for NH_2-terminal acidic residues and previous data concerning phosphorylation of β_2-AR peptides (Benovic et al., 1990), we propose that serines

369, 379, 391, 406, 412, and 416 as well as threonines 389 and 413 are likely sites of phosphorylation by β-ARK in the hamster β_2-AR. Additionally, a number of the recently cloned G protein coupled receptors contain acidic serine/threonine-rich regions in either the third intracellular loop region or the carboxyl-terminal domain (O'Dowd et al., 1989). This raises the question of whether additional receptor substrates for β-ARK may exist. Studies aimed at testing a wide variety of receptors as substrates for β-ARK as well as confirming the sites of β-ARK-mediated phosphorylation of the intact β_2-AR are currently underway.

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REFERENCES

Applebury, M. L., & Hargrave, P. A. (1986) *Vision Res. 26*, 1881–1895.

Aton, G. B., & Litman, B. J. (1984) *Exp. Eye Res. 38*, 547–559.

Benovic, J. L., Shorr, R. G. L., Caron, M. G., & Lefkowitz, R. J. (1984) *Biochemistry 23*, 4510–4518.

Benovic, J. L., Mayor, F., Jr., Somers, R. L., Caron, M. G., & Lefkowitz, R. J. (1986a) *Nature 322*, 869–872.

Benovic, J. L., Strasser, R. H., Caron, M. G., & Lefkowitz, R. J. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2797–2801.

Benovic, J. L., Regan, J. W., Matsui, H., Mayor, F., Jr., Cotecchia, S., Leeb-Lundberg, L. M. F., Caron, M. G., & Lefkowitz, R. J. (1987a) *J. Biol. Chem. 262*, 17251–17253.

Benovic, J. L., Mayor, F., Jr., Staniszewski, C., Lefkowitz, R. J., & Caron, M. G. (1987b) *J. Biol. Chem. 262*, 9026–9032.

Benovic, J. L., Kühn, H., Weyand, I., Codina, J., Caron, M. G., & Lefkowitz, R. J., (1987c) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8879–8882.

Benovic, J. L., Bouvier, M., Caron, M. G., & Lefkowitz, R. J. (1988) *Annu. Rev. Cell Biol. 4*, 405–428.

Benovic, J. L., DeBlasi, A., Stone, W. C., Caron, M. G., & Lefkowitz, R. J. (1989a) *Science 246*, 235–240.

Benovic, J. L., Stone, W. C., Caron, M. G., & Lefkowitz, R. J. (1989b) *J. Biol. Chem. 264*, 6707–6710.

Benovic, J. L., Onorato, J. J., Lohse, M. J., Dohlman, H. G., Staniszewski, C., Caron, M. G., & Lefkowitz, R. J. (1990) *Br. J. Clin. Pharmacol. 30*, 3S–12S.

Bouvier, M., Leeb-Lundberg, L. M. F., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1987) *J. Biol. Chem. 262*, 3106–3113.

Bouvier, M., Hausdorff, W. P., DeBlasi, A., O'Dowd, B. F., Kobilka, B. K., Caron, M. G., & Lefkowitz, R. J. (1988) *Nature 333*, 370–373.

Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, R., Jr. (1982) *Biochemistry 21*, 5794–5799.

Cooper, J. A., Sefton, B. M., & Hunter, T. (1983) *Methods Enzymol. 99*, 387–402.

Dohlman, H. G., Bouvier, M., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1987) *J. Biol. Chem. 262*, 14282–14288.

Fowels, C., Sharma, R. & Akhtar, M. (1988) *FEBS Lett. 121*, 139–142.

Fung, B. K. K., & Stryer, L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2500–2504.

Green, D. A., & Clark, R. B. (1981) *J. Biol. Chem. 256*, 2105–2108.

Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., & Lefkowitz, R. J. (1989) *J. Biol. Chem. 264*, 12657–12665.

Hausdorff, W. P., Caron, M. G., & Lefkowitz, R. J. (1990) *FASEB J. 4*, 2881–2889.

Hewik, R. M., Hunkapiller, M. E., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7790–7797.

Hurley, J. B., & Stryer, L. (1982) *J. Biol. Chem. 257*, 11094–11099.

Kelleher, D. J., & Johnson, G. L. (1990) *J. Biol. Chem. 265*, 2632–2639.

Kemp, B. E., Graves, D. J., Benjamini, E., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 4888–4894.

Kobilka, B. K., Matsui, H., Kobilka, T. S., Yang-Feng, T. L., Francke, U., Caron, M. G., Lefkowitz, R. J., & Regan, J. W. (1987) *Science 238*, 650–656.

Kuenzel, E. A., & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 737–741.

Kuenzel, E. A., Mulligan, J. A., Sommercorn, J., & Krebs, E. G. (1987) *J. Biol. Chem. 262*, 9136–9140.

Kühn, H., & Wilden, U. (1987) *J. Recept. Res. 7*, 283–298.

Kühn, H., Hall, S. W., & Wilden, U. (1985) *FEBS Lett. 176*, 473–478.

Kwatra, M. M., Benovic, J. L., Caron, M. G., Lefkowitz, R. J., & Hosey, M. M. (1989) *Biochemistry 28*, 4543–4547.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lefkowitz, R. J., & Caron, M. G. (1988) *J. Biol. Chem. 263*, 4993–4996.

Lohse, M., Lefkowitz, R. J., Caron, M. G., & Benovic, J. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3011–3015.

Lohse, M., Benovic, J. L., Codina, J., Caron, M. G., & Lefkowitz, R. J. (1990a) *Science 248*, 1547–1550.

Lohse, M., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1990b) *J. Biol. Chem. 265*, 3202–3209.

Marin, O., Meggio, F., Marchiori, F., Borin, G., & Pinna, L. A. (1986) *Eur. J. Biochem. 160*, 239–244.

Meggio, F., Marchiori, F., Borin, G., Chessa, G., & Pinna, L. A. (1984) *J. Biol. Chem. 259*, 14576–14579.

O'Dowd, B. F., Lekowitz, R. J., & Caron, M. G. (1989) *Annu. Rev. Neurosci. 12*, 67–83.

Palczewski, K., McDowell, J. H., & Hargrave, P. A. (1988) *Biochemistry 27*, 2306–2313.

Palczewski, K., Arendt, A., McDowell, J. H., & Hargrave, P. A. (1989) *Biochemistry 28*, 8764–8770.

Reimann, E. M., & Beham, R. A. (1983) *Methods Enzymol. 99*, 51–55.

Shichi, H., & Somers, R. L. (1978) *J. Biol. Chem. 253*, 7040–7046.

Sibley, D. R., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1987) *Cell 48*, 913–922.

Sitaramayya, A., & Liebman, P. A. (1983a) *J. Biol. Chem. 258*, 1205–1209.

Sitaramayya, A., & Liebman, P. A. (1983b) *J. Biol. Chem. 258*, 12106–12109.

Speiss, J., Rivier, J. E., Rodkey, S. A., Bennett, C. D., & Vale, W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2974–2978.

Strader, C. D., Sigal, I. S., Blate, A. D., Cheung, A. H., Register, R. B., Rands, E., Zemcik, B. A., Chandelore, M. R., & Dixon, R. A. F. (1987) *Cell 49*, 855–863.
Strader, C. D., Sigal, I. S., & Dixon, R. A. F. (1989) *FASEB J. 3*, 1825–1832.
Strasser, R. H., Sibley, D. R., & Lefkowitz, R. J. (1986) *Biochemistry 25*, 1371–1377.
Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462–477.
Thompson, P., & Findlay, J. B. C. (1984) *Biochem. J. 220*, 773–780.
Wilden, U., & Kühn, H. (1982) *Biochemistry 21*, 3014–3022.
Wilden, U., Hall, S. W., & Kühn, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1174–1178.

Stereochemistry and Size of Sugar Head Groups Determine Structure and Phase Behavior of Glycolipid Membranes: Densitometric, Calorimetric, and X-ray Studies[†,‡]

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ABSTRACT: The role carbohydrate moieties play in determining the structure and energetics of glycolipid model membranes has been investigated by small- and wide-angle X-ray scattering, differential scanning densitometry (DSD), and differential scanning microcalorimetry (DSC). The dependence of a variety of thermodynamic and structural parameters on the stereochemistry of the OH groups in the pyranose ring and on the size of the sugar head group has been studied by using an homologous series of synthetic stereochemically uniform glyceroglycolipids having glucose, galactose, mannose, maltose, or trimaltose head groups and saturated ether-linked alkyl chains with 10, 12, 14, 16, or 18 carbon atoms per chain. The combined structural and thermodynamic data indicate that stereochemical changes of a single OH group in the pyranose ring can cause dramatic alterations in the stability and in the nature of the phase transitions of the membranes. The second equally important determinant of lipid interactions in the membrane is the size of the head group. A comparison of lipids with glucose, maltose, or trimaltose head groups and identical hydrophobic moieties has shown that increasing the size of the neutral carbohydrate head group strongly favors the bilayer-forming tendency of the glycolipids. These experimental results provide a verification of the geometric model advanced by Israelachvili et al. (1980) [Israelachvili, J. N., Marcelja, S., & Horn, R. G. (1980) *Q. Rev. Biophys. 13*, 121–200] to explain the preferences lipids exhibit for certain structures. Generally galactose head groups confer highest stability on the multilamellar model membranes as judged on the basis of the chain-melting transition. This is an interesting aspect in view of the fact that galactose moieties are frequently observed in membranes of thermophilic organisms. Glucose head groups provide lower stability but increase the number of stable intermediate structures that the corresponding lipids can adopt. Galactolipids do not even assume a stable intermediate L_α phase for lipids with short chain length but perform only $L_c \rightarrow H_{II}$ transitions in the first heating. The C_2 isomer, mannose, modifies the phase preference in such a manner that only $L_\beta \rightarrow H_{II}$ changes can occur. Maltose and trimaltose head groups prevent the adoption of the H_{II} phase and permit only $L_\beta \rightarrow L_\alpha$ phase changes. The DSD studies resulted in a quantitative estimate for the volume change associated with the $L_\alpha \rightarrow H_{II}$ transition of 14-Glc. The value of $\Delta\bar{v} = 0.005$ mL/g supports the view that the volume difference between L_α and H_{II} is minute. Analogously, it was found that the enthalpy input required to transform the lamellar L_α phase into the inverted hexagonal structure is only approximately 10% of that of the $L_c \rightarrow L_\alpha$ transition.

One of the challenges of contemporary biochemistry is to unravel the roles of sugar moieties in glycoconjugates. There is a growing awareness that oligosaccharides may be instrumental in mediating such important phenomena as cell–cell recognition (Curatolo, 1987; Hakamori, 1984), interaction with toxins and viruses (Haywood, 1974; Cuatrecasas, 1973), cryoprotection (Womersley et al., 1986; Lis et al., 1990), thermostabilization of cells (Langworthy et al., 1974), and perhaps combined lipid–protein sorting in epithelial cells (Simmons & van Meer, 1988). The actual molecular mechanisms by which these reactions could proceed are far from understood, but certain possibilities suggest themselves. Single sugar moieties and more so oligosaccharides can present a remarkable variety of configurations and structures in relatively short chains by the multiple choices of isomer linkages and branching patterns. The interaction of sugars with water is highly specific and depends strongly on the stereoisomerism

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