A Family of Polypeptide Substrates and Inhibitors of Insulin Receptor Kinase†

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ABSTRACT: Previous studies have shown that reduced carbamoylmethylated lysozyme (RCAM-lysozyme, MW ~ 14.5K) is a substrate and inhibitor (K_i ~ 0.6 μM) of insulin receptor kinase (InsRK) autophosphorylation (Kohanski & Lane, 1986; Lane & Kohanski, 1986). In this study we have prepared a family of defined modified derivatives of RCAM-lysozyme and used them to probe the nature of the substrate and inhibitory sites of InsRK. All open-chain derivatives of lysozyme in which either the tryptophanyl, methionyl, cysteinyl, arginyl, or histidyl side chains were modified served as substrates and were potent inhibitors of InsRK autophosphorylation. This was true whether the substitutions were either hydrophilic or hydrophobic, although the hydrophilic derivatives had a higher inhibitory potency. Tryptic peptides derived from RCAM-lysozyme, however, were inactive as inhibitors, and a mixture of the three cyanogen bromide fragments (containing 12, 24, and 93 amino acids, respectively) was found to be less potent in inhibiting the receptor kinase. Derivatization of either tyrosyl or carboxyl side chains produced derivatives that were neither substrates nor capable of inhibiting receptor autophosphorylation. Derivatives with modified amino groups were substrates for InsRK but were not able to inhibit InsRK autophosphorylation. The present study suggests that (a) unphosphorylated InsRK has a large hydrophilic substrate binding domain and is effectively inhibited by long-chain polypeptides but not by short sequences, (b) some of the amino, carboxyl, and hydroxyphenyl side chains are essential to the inhibitory nature of these polypeptides, and (c) derivatives that fail to inhibit autophosphorylation can still be recognized and phosphorylated by active InsRK. Thus, the two mechanistically interrelated InsRK events (e.g., autophosphorylation and substrate phosphorylation) can be dissociated from one another. The study may assist in developing more potent inhibitors of InsRK autophosphorylation and in determining the ideal sequence(s) for potential physiological inhibitors, as well as for substrates of InsRK.

Insulin receptor kinase (InsRK) is an insulin-activated tyrosine-specific protein kinase [reviewed by Kahn et al. (1986)]. Insulin binding leads to autophosphorylation of the β-subunit, a phenomenon that appears to be a prerequisite for transmitting the insulin signal to metabolic pathways within the cell (Ellis et al., 1986; Chou et al., 1987). Autophosphorylation, in turn, enhances the rate of receptor-catalyzed substrate phosphorylation in vitro (Kohanski et al., 1986; Rosen et al., 1986; Yu & Czech, 1984; Morrison & Pessin, 1987; Yu et al., 1985; Rees-Jones & Taylor, 1985; White et al., 1985, 1987). Insulin binding to the α-subunit of the receptor specifically increases the V_{max} of the β-subunit kinase, thereby increasing the rates of both autophosphorylation and substrate phosphorylation.

Various tyrosyl-containing peptides have been examined as substrates of insulin receptor kinase and other tyrosine-specific protein kinases. However, most of these peptides, including those having amino acid sequences that are identical with those bordering the autophosphorylation domain of the human insulin receptor (Stadmaver & Rosen, 1983; Walker et al., 1987) or that of viral transforming proteins (Hunter, 1982), have been found to be weak substrates (K_m = 0.2–5.0 mM) and weak inhibitors for these enzymes (Hunter, 1982; Wong & Goldberg, 1983; Stadmaver & Rosen, 1983; Walker et al., 1987; Neve et al., 1981). It could be concluded from these studies, however, that the primary sequence participates in InsRK recognition of target tyrosine residues. Thus, a comparative study of tyrosyl phosphorylation sites among the viral transforming proteins has revealed the presence of a basic moiety, seven residues from the N-terminal of the phosphorylated tyrosine. In addition, one or more acidic residue (e.g., glutamic acid) are present in the intervening distance (Smart, 1981; Patchinsky & Sefton, 1981; Patchinsky et al., 1982). Thus, primary-sequence requirements do participate in the recognition of target tyrosine residues.

In contrast to tyrosyl-containing peptides, the reduced and carbamoylmethylated derivative of lysozyme (RCAM-lysozyme,[1] MW = 14.5K) has been found to be both an excellent

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[1] Abbreviations: CAMC, (carbamoylmethyl)cysteine; NEM-Cys, (*N*-ethylmaleimidyl)cysteine; oNPS-Cl, *o*-nitrobenzenesulfenyl chloride; RCAM-lysozyme, fully reduced and carbamoylmethylated derivative of lysozyme; RCAM-(NEM-Cys)$_8$-lysozyme, derivative in which the four disulfide bonds were reduced and alkylated with *N*-ethylmaleimide; RCAM-acetyl$_7$-lysozyme, RCAM-lysozyme derivative in which the seven amino groups were acetylated; RCAM-Mal$_7$-lysozyme, RCAM-lysozyme derivative in which the seven amino groups were maleilated; RCAM-Suc$_7$-lysozyme, RCAM-lysozyme derivative in which the seven amino groups were succinylated; RCAM-(NPS-Trp)$_6$-lysozyme, RCAM-lysozyme derivative in which the six tryptophanyl residues were sulfenylated to [(nitrophenyl)sulfenyl]tryptophan; RCAM-CAM-His-lysozyme, RCAM-lysozyme derivative in which the single histidyl residue was carbamoylmethylated; carboxyl-modified RCAM-lysozyme, RCAM-lysozyme derivative in which the free carboxyls were linked to glycine ethyl ester; lyzox, performic acid oxidized lysozyme; iodolyzox, lyzox derivative in which the three tyrosyl residues were iodinated; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; TNBS, trinitrobenzenesulfonic acid; NCS, *N*-chlorosuccinimide.

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substrate for InsRK and a potent inhibitor of InsRK autophosphorylation (K_i = 0.6 μM). Native lysozyme is not phosphorylated at all. RCAM-lysozyme was found to be phosphorylated exclusively on tyrosine residues (Kohanski & Lane, 1986; Lane & Kohanski, 1986), most likely on tyrosine 23 (D. Lane, personal communication). Reduction and carbamoylmethylation of the four disulfide bonds of lysozyme result in a loss of the tertiary structure of the native enzyme. This implies that the unique properties of RCAM-lysozyme, both as a substrate and as an inhibitor of InsRK, are dictated primarily by the primary sequence, namely, those amino acids surrounding the target tyrosine, and, to some extent, by the secondary (helical) structure. Since the three-dimensional structure of InsRK is still unknown, proteins and peptides of known amino acid sequences and features such as length and hydrophobicity, with a strong interaction with the phosphorylating domain of the receptor, may assist in receptor characterization. RCAM-lysozyme is perhaps the most potent inhibitor of InsRK autophosphorylation documented so far. Several copolymers containing Glu, Tyr, and Ala were tested for their inhibitory potency of InsRK autophosphorylation and found less effective (K_i = 5–10 μM; Sahal et al., 1988).

Thus in this study, we have prepared a family of defined derivatives of lysozyme, all being open-chain polypeptides. A particular group of amino acids (i.e., the six lysines, the single histidine, and the six tryptophanyl moieties) has been selectively and quantitatively modified. The potency of these derivatives in inhibiting InsRK autophosphorylation and their ability to serve as substrates were evaluated.

Experimental Procedures

Materials

Hen egg white lysozyme, bovine α-lactalbumin, iodoacetic acid, iodoacetamide, dithiothreitol, succinic anhydride, maleic anhydride, *N*-ethylmaleimide, *o*-nitrobenzenesulfenyl chloride, and trinitrobenzenesulfonic acid were purchased from Sigma (St. Louis, MO). Urea, cyanogen bromide, and hydrogen peroxide were obtained from British Drug Houses. [γ-^{32}P]-ATP was obtained from New England Nuclear. Wheat germ agglutinin–agarose was obtained from Vector Laboratories. Reagents for SDS–PAGE were supplied by Bio-Rad Laboratories. All other reagents used in this study were of analytical grade.

Preparation of Open-Chain Derivatives of Hen Egg White Lysozyme

RCAM-lysozyme. Hen egg white lysozyme (200 mg) was dissolved in 10 mL of 1 M Tris-HCl, pH 7.8–8 M urea. Dithiothreitol (30 mg) was added (5 molar excess per disulfide bond), and the reaction was allowed to proceed for 20 min at room temperature. Iodoacetamide (200 mg) was then added, and the mixture was incubated for an additional 20 min at room temperature prior to extensive dialysis (48 h) against H_2O and lyophilization.

RCAM-(NEM-Cys)$_8$-lysozyme. Lysozyme (100 mg) was dissolved in 4.0 mL of 8 M urea (pH 6.0). Dithiothreitol (20 mg) was then added, and the reaction was allowed to proceed for 10 min at room temperature. Solid *N*-ethylmaleimide (70 mg) was added. After an additional 20 min the protein derivative was extensively dialyzed against H_2O and lyophilized.

RCAM-Mal$_7$-lysozyme. RCAM-lysozyme (30 mg) was suspended in 1.5 mL of 0.01 M NaOH and cooled to 4 °C. Maleic anhydride (30 mg) was added and was dissolved by shaking. The reaction mixture was kept at pH 9.0 by the subsequent addition of NaOH for another 2 h, prior to extensive dialysis against several changes of 1 mM NaOH. The derivative was found to be trinitrobenzenesulfonic acid negative (Habeeb, 1966), indicating that essentially all the amino groups were maleilated.

RCAM-Suc$_7$-lysozyme. This was prepared with an identical procedure, substituting succinic anhydride for maleic anhydride.

RCAM-acetyl$_7$-lysozyme. RCAM-lysozyme (30 mg) was suspended in 1.5 mL of H_2O and cooled to 4 °C. Afterward, ten 15-mL aliquots of acetic anhydride were added over a period of 1 h, while the pH was kept at 7.0–7.5 by the addition of NaOH. The derivative was then dialyzed at 4 °C against 1 mM NaOH, and then for an additional 2 days against H_2O prior to lyophilization.

RCAM-(NPS-Trp)$_6$-lysozyme. RCAM-lysozyme (30 mg) was dissolved in 2 mL of 70% acetic acid; then, oNPS-Cl (40 mg) was added and dissolved by shaking. The reaction was allowed to proceed for 1 h at room temperature. The protein was then precipitated by addition of 30 mL of cold acetone–HCl (39 volumes of acetone, 1 volume of 1 M HCl), and the precipitate was collected by centrifugation, redissolved in H_2O, and dialyzed extensively. RCAM-(NPS-Trp)$_6$-lysozyme contained 6.2 mol of NPS-Trp/mol of protein as determined by its absorption at 365 nm. The molar extinction coefficient (ϵ_{365nm}) of oNPS-Trp in 70% acetic acid is 4000 (Scoffone et al., 1968; Shechter et al., 1972).

Arginine-Modified RCAM-lysozyme. The amino acid moieties of native lysozyme were first blocked with citraconic anhydride (Dixon & Perham, 1968). Lysozyme (100 mg) was dissolved in 3.0 mL of 0.1 M $NaHCO_3$ (pH 8.5) and treated with a 150-fold molar access of citraconic anhydride, added in 10 aliquots over a period of 1 h. The protein was then dialyzed against the same buffer for 24 h. The protein was found to be TNBS negative, indicating that essentially all amino side chains were blocked by this procedure. The protein was further modified at pH 10.5 with a 200-fold molar excess of 2,3-cyclohexanedione (Toi et al., 1967), added in several aliquots over a period of 7 h. The derivative was then extensively dialyzed against 0.1 M $NaHCO_3$ (pH 8.4) and then against H_2O. Next, decitraconilation was performed by incubating the protein with 0.01 M HCl for 3 h at 4 °C. The protein was then dialyzed, lyophilized, reduced, and carbamoylmethylated as described above. Amino acid analysis revealed that 95% of the arginine residues were modified in this prepration while lysine residues were unaffected.

RCAM-CNB$_r$-lysozyme. RCAM-lysozyme (30 mg) and cyanogen bromide (20 mg) were dissolved together in 1.0 mL of 70% formic acid. The reaction was allowed to proceed for 24 h at room temperature, after which the sample was diluted 10-fold with H_2O and lyophilized. The residue was then redissolved in H_2O and lyophilized twice more. RCAM-CNB$_r$-lysozyme was quantitatively cleaved to yield the appropriate-sized fragments, as judged by SDS–PAGE.

RCAM-CAM-His-lysozyme. Native lysozyme (300 mg/3.0 mL) was modified for 7 days at room temperature at pH 7.4 in the dark with 0.5 M iodoacetamide. The protein was then dialyzed extensively against H_2O and lyophilized. Reduction and carbamoylmethylation was then carried out as described above. The acid hydrolysate of this preparation was found to contain neither histidine nor cystine. Other amino acid moieties were not modified.

Tryptic (Arginyl) Peptides of RCAM-Suc$_7$-lysozyme. RCAM-Suc$_7$-lysozyme (15 mg) was suspended in 1.0 mL of 0.05 M Tris-HCl, pH 7.4, containing 1 mM $CaCl_2$. TPCK-treated trypsin [final concentration 1% (w/v)] was then added,

and digestion was allowed to proceed for 4 h at 37 °C. Most of the insoluble derivatives became solubilized after trypsinization, and the remainder was removed by centrifugation. Soybean trypsin inhibitor (4-fold molar excess over the trypsin) was later added, and the peptide mixture was frozen until used.

Carboxyl-Modified RCAM-lysozyme. RCAM-lysozyme (15 mg) was dissolved in 1.0 mL of 1 M glycine ethyl ester. The pH was adjusted to 5.5, and the 3-mg aliquots of EDC were added over a period of 5 h, after which the protein derivative was dialyzed extensively for 3 days against H_2O and then lyophilized.

RCAM-(oxindolyl-Ala)$_6$-lysozyme. The tryptophanyl moieties of RCAM-lysozyme (5 mg/mL in 0.1 M acetic acid) were oxidized to oxindolylalanine with *N*-chlorosuccinimide (Shechter et al., 1975). Aliquots of solid NCS were added to the protein solution, and the reaction was followed by the reduction in absorbance at 280 nm and terminated when no further reduction in absorbance was observed. The derivative was dialyzed against H_2O, lyophilized, and loaded on a Sephadex-G-25 column (40 × 1 cm). The protein peak was pooled and lyophilized. RCAM-(oxindolyl-Ala)$_6$-lysozyme had molar extinction coefficient ϵ_{280nm} = 15 200. The methionine residues of this derivative are modified as well (see footnotes of Table I).

Performic Acid Oxidized Lysozyme (Lyzox). Performic acid [95% formic acid–5% H_2O_2 (v/v)] was prepared and allowed to incubate at room temperature for 1 h. Lysozyme (40 mg) was dissolved in 2 mL of the preequilibrated performic acid. The mixture was incubated for 1 h at room temperature, diluted 10-fold with cold H_2O, dialyzed extensively for 3 days against H_2O, and then lyophilized.

Iodolyzox. Lyzox (15 mg) was dissolved in 1.0 mL of 0.05 M Tris-HCl, pH 7.4, containing NaI (20 mM). Ten aliquots of chloramine T (0.4 mg each) were then added over a period of 1 h (4-fold molar excess per tyrosyl residue). The protein derivative was dialyzed extensively against H_2O and finally lyophilized.

All lysozyme derivatives were carefully characterized by amino acid analysis after acid hydrolysis and by spectrophotometrical methods. For the sake of brevity, detailed analysis of each derivative is not presented here. The derivatives in which the amino acid side chains were blocked or in which the tryptophans were modified failed to yield a colored reaction with trinitrobenzenesulfonic acid or with oNPS-Cl, respectively (Habeeb, 1966; Scoffone et al., 1968). Amino acid analysis of RCAM-lysozyme revealed that it lacked cystine residues but contained 7.2 mol of CMC/mol of derivative; lyzox itself contained 7.7 mol of cysteic acid per mole. The hydrolysate of the carboxyl-modified RCAM-lysozyme was found to contain an additional ~10.3 mol of glycine/mol of derivative. Additional details concerning the characterization of the derivatives are given in the footnotes of Table I.

Other Procedures

Preparation of Solubilized Insulin Receptor. Rats were killed with carbon monoxide, and the livers were removed, washed with ice-cold saline, weighed, and minced with scissors. Ten volumes of 50 mM HEPES (pH 7.4), 0.25 M sucrose supplemented with aprotinin (10 μg/mL), and 2 mM phenylmethanesulfonyl fluoride was added to 6 g of liver tissue, and the mixture was homogenized in a Dounce homogenizer with a loose-fitting pestle. The homogenate was centrifuged at 1200 rpm in a Sorvall centrifuge to remove large clumps, and Triton X-100 was added to the supernatant to a final concentration of 1%. The resulting suspension was stirred for 60 min at 4 °C and then centrifuged at 100000*g* for 60 min, and the supernatant was applied to a 2-mL column of agarose-bound wheat germ agglutinin (WGA) at 4 °C. The column was washed extensively with 50 mM HEPES buffer (pH 7.4) containing 0.1% Triton X-100, and the receptor eluted with this buffer supplemented with 0.3 M *N*-acetyl-D-glucosamine.

In Vitro Autophosphorylation Assay. WGA-purified insulin receptor (5 μL from 80 μg/mL of total protein) was incubated at 22 °C for 20 min in a solution containing 50 mM HEPES and 5 mM $MnCl_2$, in the absence or the presence of 100 nM insulin, and the indicated concentration of the various lysozyme derivatives in a final volume of 50 μL. Phosphorylation was initiated by the addition of 25 μM [γ-^{32}P]ATP (1 μCi) to the solution. After 2 min the reaction was stopped by addition of 5× electrophoresis sample buffer containing 100 mM dithiothreitol and boiling for 3 min. The phosphoproteins were then separated by SDS–PAGE under reducing conditions on 7.5% gels.

In Vitro Phosphorylation of Protein Substrates. Solubilized lectin-purified insulin receptor aliquots (3.6 μg of protein each) were diluted to a final volume of 50 μL in a solution containing 50 mM HEPES (pH 7.0), 0.1% Triton X-100, and 5 mM Mn^{2+}. These mixtures were preincubated, either with or without insulin (10^{-7} M), at 22 °C for 20 min and then further incubated at 22 °C for 10 min with the addition of 50 μM ATP to allow receptor autophosphorylation. [γ-^{32}P]ATP and varying concentrations of the various lysozyme derivatives were then added, and the incubations were continued for 5 min. The reactions were stopped by addition of 5× electrophoresis sample buffer containing 100 mM dithiothreitol and boiling for 3 min. The phosphorylated derivatives were then separated by SDS–PAGE under reducing conditions on 12.5% gels.

RESULTS

Preparation of Open-Chain Derivatives of Lysozyme. RCAM-lysozyme, but not native lysozyme, inhibited InsRK autophosphorylation over a concentration range of 0.1–10 μM. Therefore, we have prepared and screened open-chain lysozyme derivatives in which the disulfide bonds were either reduced or oxidized and the native structure was disrupted. The derivatives studied are listed in Table I. Special precautions were taken in order to achieve both quantitation and selectivity toward specific residues. All derivatives were analyzed by both amino acid and spectrophotometric analyses as specified under Experimental Procedures and in Table I. Our experimental protocol and the source of the insulin receptors differed from those used by Kohanski and Lane (1986). Nevertheless, essentially the same K_i value (0.45 versus 0.6 μM) for InsRK inhibition by RCAM-lysozyme was obtained in our study (Figure 1, Table II) and in the study of Kohanski and Lane (1986), respectively. Each set of experiments included RCAM-lysozyme as a positive control. All lysozyme derivatives used here were as soluble or more soluble than RCAM-lysozyme and remained in solution under the experimental conditions used.

Length of the Inhibitory Polypeptide Chain. The inhibitory effect of RCAM-lysozyme on InsRK autophosphorylation depended on the chain length of the peptide used. The mixture of the three cyanogen bromide fragments of RCAM-lysozyme (containing 12, 24, and 93 amino acids, respectively) was still a potent inhibitor, being about 7-fold less potent than RCAM-lysozyme in inhibiting InsRK autophosphorylation (Table II). In contrast, the tryptic (arginyl) peptides of RCAM-succinylated lysozyme did not inhibit auto-

Table I: Type and Number of Modified Amino Acid Moieties in Lysozyme Derivatives

derivative designation	type and no. of residues modified in addition to cystine
RCAM-lysozyme[a]	
RCAM-$(NEM\text{-}Cys)_8$-lysozyme[b]	
RCAM-$acetyl_7$-lysozyme[c]	lysine (6)
RCAM-Mal_7-lysozyme[d]	lysine (6)
RCAM-CNB_r-lysozyme[e]	methionine (2)
RCAM-$(NPS\text{-}Trp)_6$-lysozyme[f]	tryptophan (6)
arginine-modified RCAM-lysozyme[g]	arginine (11)
carboxyl-modified RCAM-lysozyme[h]	aspartic (10) and glutamic (2) acids
RCAM-CAM-His-lysozyme[i]	histidine (1)
RCAM-$(oxindolyl\text{-}Ala)_6$-lysozyme[j]	tryptophan (6), methionine (2)
lyzox[k]	tryptophan (6), methionine (2)
iodolyzox[l]	tryptophan (6), methionine (2), tyrosine (3)

[a] Acid hydrolysate contains no cystine and 7.7 mol of CMC. [b] Acid hydrolysate contains no cystine. [c] Derivative is trinitrobenzenesulfonic acid negative. [d] Derivative is trinitrobenzenesulfonic acid negative. [e] Acid hydrolysate contains no methionine; cleavage with cyanogen bromide is quantitative as judged by SDS gel electrophoresis. [f] Derivative contains 6.2 mol of NPS-Trp/mol of lysozyme as judged by its absorbance at 365 nm (ϵ_{365nm} = 4000 in 80% acetic acid; Shechter et al., 1972). [g] Acid hydrolysate contains 0.6 mol of arginine/mol of derivative only; 95% of the arginines were modified. [h] Acid hydrolysate contains an additional 10.4 mol of glycine/mol of lysozyme. [i] Acid hydrolysate contains no histidine. [j] Derivative does not react with the tryptophanyl-specific reagent oNPS-Cl (Scoffone et al., 1968). Contains 1.8 mol of methionine sulfoxide/mol of protein [estimated according to Shechter et al. (1975)]. [k] Acid hydrolysate contains 7.7 mol of cysteic acid and 1.9 mol of methionine sulfoxide per mole of lysozyme [methionine sulfoxide was estimated according to Shechter et al. (1975)]. Derivative does not react with oNPS-Cl and has a molar extinction coefficient ϵ_{280nm} = 19 500 (native lysozyme has ϵ_{280nm} = 39 000). [l] Derivative does not incorporate radioactive iodine by the chloramine T method.

phosphorylation (Table II). This implies that InsRK has an extended binding site to accomodate relatively large polypeptide chains and shorter fragments, which are likely to have a lower affinity and are thus less effective inhibitors of InsRK autophosphorylation.

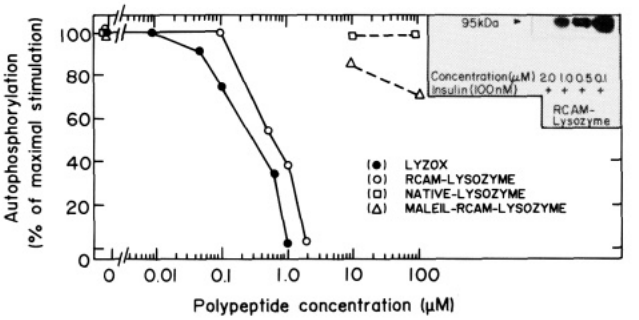

FIGURE 1: Inhibition of autophosphorylation with polypeptide derivatives of lysozyme. Partially purified insulin receptor was incubated with [γ-^{32}P]ATP in the absence or the presence of 1×10^{-7} M insulin and increasing concentrations of the polypeptide derivatives for 20 min at 22 °C essentially as described in Table II. The reaction mixture was stopped by boiling the samples, and then they were applied to 7.5% SDS–PAGE under reducing conditions. The amount of radioactivity incorporated into the β-subunit in each lane was evaluated both by densitometric analysis and by cutting out the bands and counting their radioactive content. (Inset) Autoradiogram of SDS–PAGE (7.5%) at varying concentrations of RCAM-lysozyme.

Cysteinyl and Tryptophanyl Moieties. Quantitative derivatization of the eight cysteinyl residues, to either (carbamoylmethyl)cysteine (a hydrophilic moiety) or NEM-Cys (a hydrophobic side chain), produced derivatives capable of inhibiting InsRK autophosphorylation (Table III). Additional derivatization of the six tryptophanyl moieties of RCAM-lysozyme produced a derivative also capable of inhibiting InsRK. Thus, neither cysteinyl nor intact indole moieties seem to participate directly in the receptor–polypeptide interaction. The introduction of hydrophobic side chains as opposed to hydrophilic ones produced derivatives having somewhat reduced inhibitory potency (i.e., RCAM-$(NEM\text{-}Cys)_8$-lysozyme versus RCAM-lysozyme, Table III). Thus, the extended binding pocket of InsRK appears hydrophilic in nature. This point is further substantiated in subsequent paragraphs.

Histidyl and Arginyl Moieties. Carbamoylmethylation of the single histidyl residue of RCAM-lysozyme also produced a derivative capable of inhibiting InsRK autophosphorylation (Table IV). Similarly, hydrophobic modification of the arginyl moieties of RCAM-lysozyme reduced, but did not abolish, the inhibitory potency of this derivative (Table IV).

Table II: Inhibitory Potency of InsRK Autophosphorylation by Native Lysozyme, RCAM-lysozyme, and RCAM-lysozyme Fragments

derivative designation	description (remarks)	inhibition of InsRK autophosphorylation, K_i (μM)[a]
native lysozyme	disulfide bonded	>100
RCAM-lysozyme	open chain	0.45 ± 0.1
RCAM-CNB_r-lysozyme	open chain; three fragments, MW ~ 1K, 3K, and 10K	2.6 ± 0.3
arginyl (tryptic) peptides of RCAM-Suc_7-lysozyme	short peptides	>100

[a] Wheat germ agglutinin purified insulin receptor (4 μg/assay) was incubated with insulin (100 nM) in solution containing 50 mM HEPES and 5 mM $MnCl_2$ and increasing concentrations of the various lysozyme derivatives in a final volume of 50 μL. Phosphorylation was initiated by the addition of 25 μM [γ-^{32}P]ATP (1 μCi) to the solution. After 2 min the reaction was stopped by addition of 5× electrophoresis sample buffer containing 100 mM DTT and boiling for 3 min. The samples were run on SDS–PAGE. The gel was dried out, and autoradiography was performed. The extent of phosphorylation was measured both by densitometric analysis and by cutting out the bands and measuring them for their radioactive content. Each lysozyme derivative was examined for its inhibitory potency in at least three separate experiments. Values are the mean ± SEM.

Table III: Inhibitory Potency of Open-Chain Derivatives Having Modified Cystines and Tryptophanyl Side Chains[a]

derivative designation	modification	character	inhibition of InsRK autophosphorylation, K_i (μM)
RCAM-lysozyme	(carbamoylmethyl)cysteine	hydrophilic	0.45 ± 0.1
RCAM-$(NEM\text{-}Cys)_8$-lysozyme	NEM-Cys	hydrophobic	3 ± 0.7
RCAM-$(oxindolylAla)_6$-lysozyme	oxindolylalanine	hydrophilic	3 ± 1.0
RCAM-$(NPS\text{-}Trp)_6$-lysozyme	[(*o*-nitrophenyl)sulfenyl]tryptophan	hydrophobic	5 ± 1.0

[a] The derivatives listed were all tested under conditions identical with those described in Table I.

Table IV: Role of Polypeptide and Histidyl, Arginyl, Lysyl, Carboxyl, and Tyrosyl Side Chain(s) in Inhibiting Insulin Receptor Kinase Autophosphorylation[a]

derivative designation	type and no. of modified amino acids (other than cystines)	inhibition of InsRK autophosphorylation, K_i (μM)
RCAM-lysozyme	none	0.45 ± 0.1
RCAM-CAM-His-lysozyme	histidine (1)	2.0 ± 0.2
arginine-modified RCAM-lysozyme	arginine (11)	8 ± 2
RCAM-Mal$_7$-lysozyme	lysine (6)	>100
RCAM-acetyl$_7$-lysozyme	lysine (6)	>100
carboxyl-modified RCAM-lysozyme	glutamic (8) and aspartic (2) acids	>100

[a] The derivatives listed were all tested under conditions identical with those described in Table I.

Table V: Inhibitory Potency of Lyzox and Tyrosine-Modified RCAM-lysozyme toward InsRK Autophosphorylation[a]

derivative designation	type and no. of modified amino acids	inhibition of InsRK autophosphorylation, K_i (μM)
RCAM-lysozyme	half-cystine (8)	0.45 ± 0.1
lyzox	half-cystine (8) tryptophan (6) methionine (2)	0.25 ± 0.1
iodolyzox	half-cystine (8) tryptophan (6) methionine (2) tyrosine (3)	>100

[a] The derivatives listed were all tested under conditions identical with those described in Table I and under Experimental Procedures.

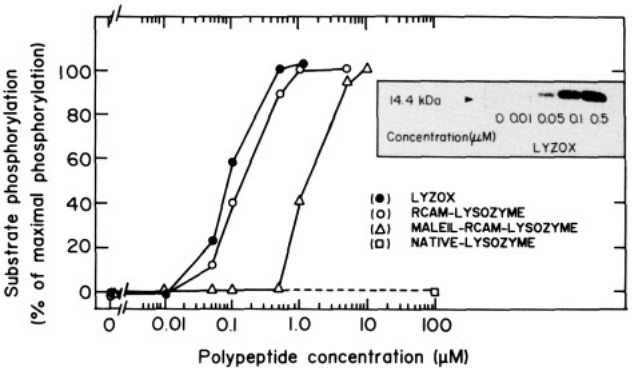

FIGURE 2: Phosphorylation of the polypeptide derivatives by activated InsRK. Partially purified insulin receptor was preincubated with 10^{-7} M insulin and 25 μM ATP to allow receptor autophosphorylation. [γ-^{32}P]ATP and the indicated concentrations of the polypeptide derivatives were then added. The reaction was carried out for 10 min at 22 °C and then terminated by boiling in 5× electrophoresis sample buffer. Samples were separated on 12.5% SDS–PAGE under reducing conditions. (Inset) The radioactive content in each lane was evaluated both by densitometric analysis and by cutting out the corresponding bands and measuring their radioactive content directly.

Thus, the imidazole group of histidine and the guanidino groups of arginines do not appear to participate directly in the inhibitor–receptor interaction.

Lysyl and Carboxyl Moieties. RCAM-lysozyme derivatives in which the amino groups were either acetylated or maleilated do not inhibit InsRK autophosphorylation. Also, selective derivatization of the carboxylic side chains of aspartic and glutamic acids of RCAM-lysozyme produced a derivative devoid of inhibitory action (Table IV). Thus, intact amino group(s) and free carboxyl(s) are essential for both the interaction with and the inhibition of InsRK. Interestingly, the amino-modified derivative that does not inhibit InsRK autophosphorylation can still serve as a substrate for the insulin receptor (see below).

Inhibitory Potency of Other Derivatives and Role of Tyrosyl Residues. Lyzox (performic acid oxidized lysozyme) was found to be either equipotent or somewhat more potent than RCAM-lysozyme in inhibiting InsRK. The K_i for the inhibition was 0.25 ± 0.1 μM (Table V, Figure 1). In this derivative, the cysteinyl residues were oxidized to cysteic acid, the tryptophanyl moieties to *N*-formylkinurenine, and the methionyl residues to methionine sulfoxide. All of these derivatizations are hydrophilic in nature and significantly increase the hydrophilicity of the polypeptide.

Iodination of the three tyrosyl residues of lyzox produced a derivative devoid of the ability to inhibit InsRK autoiphosphorylation (Table V). Thus, intact tyrosyl(s) (in addition to nonaltered amino and carboxyl moieties) appear(s) to be essential for inhibition of InsRK autophosphorylation.

Substrate Properties of the Various Derivatives. All open-chain derivatives having both nonmodified tyrosyl and free carboxylic side chains were found capable of serving as substrates for InsRK (see representative derivatives in Table VI and Figure 2). Of great interest are the RCAM-lysozyme derivatives in which the amino groups were modified (i.e., RCAM-Mal$_7$-lysozyme). This derivative can still serve as a substrate for phosphorylation but does not inhibit InsRK au-

Table VI: Potency of the Various Lysozyme Derivatives To Serve as Substrates for Activated InsRK[a]

derivative	type and no. of residues modified in addition to cystine	derivative concn required for half-maximal phosphorylation (μM)
RCAM-lysozyme		0.08
lyzox	tryptophan (6) methionine (2)	0.06
RCAM-Mal$_7$-lysozyme	lysine (6)	2
iodolyzox	tryptophan (6) methionine (2) tyrosine (3)	>40
carboxyl-modified RCAM-lysozyme	glutamic (8) and aspartic (2) acids	>40

[a] Wheat germ agglutinin purified insulin receptor aliquots (3.6 μg of protein each, in 50 μL of 50 mM HEPES, pH 7.0, 0.1% Triton X-100, and 5 mM Mn^{2+}) were incubated with 10^{-7} M insulin for 20 min and then incubated for 10 min with 50 μM ATP to allow receptor autophosphorylation. [γ-^{32}P]ATP and varying concentrations of the various lysozyme derivatives were then added, and the incubations were continued for 5 min. The reaction was stopped by addition of 5× electrophoresis sample buffer containing 100 mM DTT and boiling for 3 min. The phosphorylated derivatives were then separated on 12.5% SDS–PAGE, and the extent of phosphorylation was measured by densitometry and by direct counting of the sliced bands.

1 Lys-Val-Phe-Gly-Arg-Camc-Glu-Leu-Ala-Ala-Ala-Met-Lys-Arg-
15 His-Gly-Leu-Asp-Asn-Tyr-Arg-Gly-Tyr-Ser-Leu-Gly-Asn-Trp
29 Val-Camc-Ala-Ala-Lys-Phe-Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala
43 Thr-Asn-Arg-Asn-Thr-Asp-Gly-Ser-Thr-Asn-Tyr-Gly-Ile-Leu
57 Glu-Ile-Asn-Ser-Arg-Trp-Trp-Camc-Asn-Asp-Gly-Arg-Thr-Pro
71 Gly-Ser-Arg-Asn-Leu-Camc-Asn-Ile-Pro-Camc-Ser-Ala-Leu-Leu
85 Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-Camc-Ala-Lys-Lys-Ile
99 Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp-Val-Ala-Trp-Arg
113 Asn-Arg-Camc-Lys-Gly-Thr-Asp-Val-Gln-Ala-Trp-Ile-Arg-Gly
127 Camc-Arg-Leu

FIGURE 3: Amino acid sequence of RCAM-lysozyme. Some of the amino acid moieties that were found in this study to be essential for inhibiting InsRK autophospohorylation are underlined. Camc, (carbamoylmethyl)cysteine.

tophosphorylation at all (Table IV), implying that these two mechanistically interrelated phosphorylation events can be examined independently of each other. It seems that nonphosphorylated InsRK recognizes and interacts strongly with a polypeptide having intact tyrosyl(s), carboxyl(s) and amino group(s). Other side chains appear to be less essential for this interaction. In contrast, phosphorylated (e.g., activated) InsRK appears to have less stringent requirements, needing only intact carboxyl groups in addition to tyrosyl phosphorylation sites.

Discussion

During the last decade, both the amino acid sequence and several key functions of the InsRK were elucidated [for reviews, see Czech (1985) and Kahn et al. (1986)]. In contrast, data concerning the teritary structure of the molecule and its role in InsRK activity have been lacking. In this respect, polypeptide inhibitors of InsRK, particularly those which interact directly with the InsRK autophosphorylation domain(s), may assist in understanding the structure–function relationships within InsRK. Such a polypeptide is RCAM-lysozyme (Kohanski & Lane, 1986; Lane & Kohanski, 1986; Figure 1; Table II; see also Figure 3 for the amino acid sequence of RCAM-lysozyme). In this study we have prepared a family of modified open-chain derivatives of lysozyme and studied their inhibitory potency on InsRK autophosphorylation. We have concluded the following: D (a) The autophosphorylation site appears to be an expanded domain which can accommodate a polypeptide of 130 amino acids. A smaller fragment (i.e., the 93 amino acid cyanogen bromide fragment) binds less effectively, and shorter peptides do not seem to bind at all to the phosphorylation site of nonphosphorylated InsRK. (b) The binding pocket is hydrophilic in nature. (c) The minimal essential requirements of the inhibitory polypeptide molecule include nonaltered amino, carboxylic, or hydroxyphenyl moieties. (d) Other side chains do not participate in the binding directly, although hydrophilic substitutions can enhance polypeptide binding to the hydrophilic pocket of InsRK. (e) Amino side chain blocked derivatives which failed to inhibit autophosphorylation are both recognized and phosphorylated by activated (autophosphorylated) InsRK. Our data are consistent with the hypothesis that assumes the existence of either two distinct binding domains of InsRK or a single domain, which could be altered conformationally (most likely due to autophosphorylation) toward a substrate-specific domain. The types of amino acids that include the residue(s) essential to the inhibitory potential of the polypeptide molecule are shown in Figure 3. On the basis of previous studies, the putative domain of the polypeptide sequence most essential

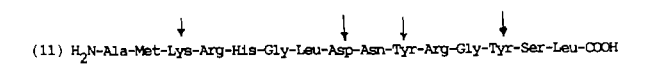

FIGURE 4: Putative more essential region of RCAM-lysozyme for inhibiting InsRK autophosphorylation. The essential amino acids as identified in this study are labeled by arrows.

for InsRK inhibition (amino acids 11–25 of lysozyme) is drawn in Figure 4. This sequence, which partly resembles the consensus autophosphorylation sites of several viral and mammalian tyrosine kinases (Hunter, 1982; Wong et al., 1983; Stadmaver & Rosen, 1983; Walker et al., 1987), includes two (of the three) tyrosine residues, a lysine residue, seven amino acid residues proximal to the N-terminal site of tyrosine 20, and aspartic acid in the intervening sequence (Figure 4). As concluded in this study, however, this putative domain should be a part of a longer polypeptide chain to effectively inhibit InsRK autophosphorylation.

Hormone-dependent cascades are usually under the influence of multiple regulatory levels (Ross & Gilman, 1980; Schramm & Selinger, 1984). It now appears that several members of the Ser/Thr protein kinase family contain homologous catalytic domains which are coupled with regulatory domains and can be either present on separate subunits (e.g., protein kinase A) or fused together on the same subunit (as in most other cases). Therefore, constitutively active catalytic domains can be generated either by dissociation or by limited proteolysis (Kemp et al., 1987; Hardie, 1988; Corbin et al., 1978; Pearson et al., 1988). The ability of certain polypeptide sequences to act as potent inhibitors of InsRK autophosphorylation raises the question whether or not tyrosine kinases (such as InsRK itself) are also under the regulatory influence of such polypeptide(s) in vivo. This and related issues raised above are to be studied.

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References

Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A., & Rosen, O. M. (1987) *J. Biol. Chem. 262*, 1842–1847.

Corbin, J. D., Sudgen, P. H., West, L., Flockhart, D. A., Lincoln, T. M., & McCarthy, D. (1978) *J. Biol. Chem. 253*, 3997–4003.

Czech, M. P. (1985) *Annu. Rev. Physiol. 47*, 357–381.

Dixon, H. B. F., & Perham, R. N. (1968) *Biochem. J. 109*, 312–320.

Ellis, L., Clausen, E., Morgan, D. O., Edery, M., Roth, R. A., & Rutter, W. J. (1986) *Cell 45*, 721–732.

Habeeb, A. F. S. A. (1966) *Anal. Biochem. 14*, 328–335.

Hardie, G. (1988) *Nature 335*, 592–593.

Hunter, T. (1982) *J. Biol. Chem. 258*, 1022–1025.

Kahn, C. R., Grigorescu, F., Takayama, S., & White, M. F. (1986) *The Role of Receptors in Biology and Medicine* (Gotto, A. M., & O'Malley, B., Eds.) pp 137–160, Raven Press, New York.

Kemp, B. E., Pearon, R. B., Gurriero, V., Bagchi, I. C., & Means, A. R. (1987) *J. Biol. Chem. 262*, 2542–2548.

Kohanski, R. A., & Lane, D. M. (1986) *Biochem. Biophys. Res. Commun. 134*, 1312–1318.

Kohanski, R. A., Frost, S. C., & Lane, M. D. (1986) *J. Biol. Chem. 261*, 12272–12281.

Lane, D. M., & Kohanski, R. A. (1986) *Mechanisms of Insulin Action* (Belfrage, P., Donner, J., & Stalfors, P., Eds.) pp 59–73, Elsevier Science Publishers, Amsterdam.

Morgan, D. O., & Roth, R. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 41-45.
Morrison, B. D., & Pessin, J. E. (1987) *J. Biol. Chem. 262*, 2861-2868.
Neil, J. C., Ghysdael, J., Vogt, P. K., & Smart, J. (1981) *Nature 291*, 675-677.
Patchinsky, T., & Sefton, B. (1981) *J. Virol. 39*, 104-114.
Patchinsky, T., Hunter, T., Esch, F. S., Cooper, J. A., & Sefton, B. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 973-977.
Pearson, R. B., Wettenhall, R. E. H., Means, A. R., Hartshome, D. J., & Kemp, B. E. (1988) *Science 241*, 970-973.
Rees-Jones, R. W., & Taylor, S. C. (1985) *J. Biol. Chem. 260*, 4461-4467.
Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M., & Cobb, M. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3237-3240.
Ross, E. M., & Gilman, A. G. (1980) *Annu. Rev. Biochem. 49*, 533-564.
Sahal, D., Ramachandran, J., & Fujita-Yamaguchi, Y. (1988) *Arch. Biochem. Biophys. 260*, 416-426.
Schramm, M., & Selinger, Z. (1984) *Science 225*, 1350-1356.
Scoffone, E., Fontana, A., & Rocchi, R. (1968) *Biochemistry 7*, 971-979.
Shechter, Y., Burstein, Y., & Patchornik, A. (1972) *Biochemistry 11*, 653-660.
Shechter, Y., Burstein, Y., & Patchornik, A. (1975) *Biochemistry 14*, 4497-4503.
Smart, J. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6013-6017.
Stadtmaver, L. A., & Rosen, O. M. (1983) *J. Biol. Chem. 258*, 6682-6685.
Toi, K. E., Bynum, E., Norris, E., & Itano, H. A. (1967) *J. Biol. Chem. 242*, 1036-1043.
Walker, D. H., Kupuswamy, D., Visvanathan, A., & Pike, L. J. (1987) *Biochemistry 26*, 1428-1433.
White, M. F., Maron, R., & Kahn, C. R. (1985) *Nature 318*, 183-186.
White, M. F., Stegman, E. W., Dull, T. J., Ullrich, A., & Kahn, C. R. (1987) *J. Biol. Chem. 262*, 9769-9777.
Wong, T. W., & Goldberg, A. R. (1983) *J. Biol. Chem. 261*, 10000-10005.
Yu, K. T., & Czech, M. P. (1984) *J. Biol. Chem. 259*, 5277-5286.

Yeast Allosteric Chorismate Mutase Is Locked in the Activated State by a Single Amino Acid Substitution†

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ABSTRACT: Chorismate mutase, a branch-point enzyme in the aromatic amino acid pathway of *Saccharomyces cerevisiae*, and also a mutant chorismate mutase with a single amino acid substitution in the C-terminal part of the protein have been purified approximately 20-fold and 64-fold from overproducing strains, respectively. The wild-type enzyme is activated by tryptophan and subject to feedback inhibition by tyrosine, whereas the mutant enzyme does not respond to activation by tryptophan nor inhibition by tyrosine. Both enzymes are dimers consisting of two identical subunits of M_r 30000, each one capable of binding one substrate and one activator molecule. Each subunit of the wild-type enzyme also binds one inhibitor molecule, whereas the mutant enzyme lost this ability. The enzyme reaction was observed by ^{1}H NMR and shows a direct and irreversible conversion of chorismate to prephenate without the accumulation of any enzyme-free intermediates. The kinetic data of the wild-type chorismate mutase show positive cooperativity toward the substrate with a Hill coefficient of 1.71 and a $[S]_{0.5}$ value of 4.0 mM. In the presence of the activator tryptophan, the cooperativity is lost. The enzyme has an $[S]_{0.5}$ value of 1.2 mM in the presence of 10 μM tryptophan and an increased $[S]_{0.5}$ value of 8.6 mM in the presence of 300 μM tyrosine. In the mutant enzyme, a loss of the cooperativity was observed, and $[S]_{0.5}$ was reduced to 1.0 mM. This enzyme is therefore locked in the activated state by a single amino acid substitution.

The binding of an effector at an allosteric site of an enzyme with the induction of a conformational change appears to form the basis for many aspects of regulation. Most allosteric enzymes are oligomeric, and binding of allosteric effectors leads to an altered conformational state having a different binding constant for effectors. One model describing this conformational change was proposed by Monod et al. (1965). According to this model, an allosteric enzyme consists of an equilibrium of a T (tense) state, having a lower substrate affinity, and an R (relaxed) state, having a higher substrate affinity. Increasing substrate concentrations pull the T-R equilibrium toward the R state with a higher substrate affinity, which results in a sigmoidal substrate saturation curve (positive cooperativity). An activator, however, transforms the equilibrium toward the R state, resulting in an equal binding

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