Rat Liver Polysome N^{α}-Acetyltransferase: Substrate Specificity†

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Received June 29, 1990; Revised Manuscript Received November 5, 1990

ABSTRACT: The substrate specificity of polysome rat liver N^{α}-acetyltransferase (NAT) has been examined by utilizing a series of synthetic and natural substrates that has been systematically altered with respect to N-terminal sequence and length. Families of peptides of the structure S-Y-S-G-G-L-L-L were generated by successively replacing the N-terminal serine, the penultimate tyrosine, and the antepenultimate serine with all 19 commonly occurring amino acids, which were then assessed for their reactivity with the rat liver enzyme. Only peptides with N-terminal serine, alanine, methionine, leucine, and phenylalanine were modified. Glycine, lysine, arginine, valine, isoleucine, and tryptophan in the second position are (with N-terminal serine) strongly inhibitory, and proline completely blocks modification. Third-position substitutions have less of an effect on NAT activity with glycine, aspartic acid, glutamic acid, and tryptophan being most inhibiting (with N-terminal Ser-Tyr). These observations are generally in agreement with in situ modifications although there are some significant differences particularly with respect to the amino-terminal residues. Optimal chain length was determined to be 10–11 residues with either synthetic peptides of the structure S-Y-S-$(G)_n$-L-L-L or adrenocorticotropin (ACTH) sequences ranging from 8 to 39 residues. The ACTH peptides were generally found to be severalfold better substrates than the corresponding synthetic ones. Activity was not affected by increased chain length beyond ~17 residues. These data support the view that polysome-catalyzed N^{α}-acetylation occurs as a cotranslational event on nascent chains of about 20–40 amino acids in length.

The combined action of ribosomal methionine aminopeptidase (MAP)[1] and N^{α}-acetyltransferase (NAT) leads to four classes of proteins, formed cotranslationally, that are distinguished by their N-terminal structures (Arfin & Bradshaw, 1988). As a result, N^{α}-acetylation can occur on some proteins with or without the initiator methionine but not on others. The selectivity of these modifications appears to reside primarily in the sequence of amino acids immediately adjacent to the methionine residue, as judged by both direct measurements (Huang et al., 1987; Boissel et al., 1988) and by examination of existing structures (Driessen et al., 1985; Persson et al., 1985). Sherman et al. (1986) proposed that the specificity of eukaryotic (yeast) MAP is governed by the size of the side chain of the penultimate residue; a similar suggestion has been made for prokaryotic (*Escherichia coli*) MAP (Hirel et al., 1989). The observed specificity of ribosomal NAT, which apparently acts after MAP, appears to be governed more by the nature of the amino-terminal residue. Of the penultimate residues exposed by MAP, alanine, serine, threonine, and glycine are generally found to be acetylated (Driessen et al., 1985; Persson et al., 1985). In addition, the initiator methionine can also be acetylated; this generally occurs when the penultimate residue has an acid or amide side chain (Huang et al., 1987; Boissel et al., 1988). However, it has also been observed that several cytoplasmic proteins possessing these N-terminal residues are not acetylated (Gonda et al., 1989) and that some N-acetylated termini do not fit this pattern, suggesting that NAT must also recognize some additional sequences or conformational features within the N-terminal region that can affect substrate specificity. Recent studies with purified NAT enzymes from yeast (Lee et al., 1988) and hen oviduct (Kamitani et al., 1989) have shown that these variations, observed in situ, are the same as those arising from direct NAT catalysis.

In the accompanying paper, we reported the isolation and characterization of rat liver polysomal NAT (Yamada & Bradshaw, 1991). This enzyme shares many of the physical properties of the yeast enzyme (Lee et al., 1988) but is quite different, particularly in subunit structure, from hen oviduct NAT (Kamitani et al., 1989; Kamitani & Sakiyama, 1989), the only other animal form of the enzyme isolated to date. A study on the specificity of the yeast enzyme has been reported (Lee et al., 1990b), but little information is available regarding the specificity of the hen enzyme. In this paper, using a series of 65 synthetic peptides, we describe the selectivity of rat liver polysome NAT with respect to substrate sequence and chain length.

Experimental Procedures

Materials. Most of the synthetic peptides were prepared by solid-phase techniques using an ABI Model 430A peptide synthesizer programmed for t-Boc chemistry. Deprotection and cleavage from the resin were achieved by HF methodology and were carried out on prepared resin samples provided to Immuno-Dynamics, Inc., La Jolla, CA. The remaining peptides were obtained from Multiple Peptide Systems, Inc., San Diego, CA. Adenocorticotropin (ACTH) fragments 1–10, 1–13, 1–17, 1–24, and 1–39 were obtained from Sigma, St. Louis, MO. ACTH-1–8 (or des-Ac-α-MSH-1–8) were used as the routine substrate and was obtained from Geoff Tregear, Howard Florey Institute, Melbourne, Australia, as described

† Supported by USPHS Research Grant DK 32465. We thank the Hitachi Chemical Co., Ltd., of Japan for their generous support of R.Y. throughout this work.

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[1] Abbreviations: MAP, methionine aminopeptidase; NAT, N^{α}-acetyltransferase; MSH, melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone; HPLC, high-performance liquid chromatography; ODS, octadecylsilane; t-Boc, *tert*-butoxycarbonyl.

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Table I: Effect of Amino Acid Substitution in Positions 1, 2, and 3 on the N^{α}-Acetylation of Peptide S-Y-S-G-G-L-L-L- by Rat Liver Polysome N^{α}-Acetyltransferase[a]

	relative activity (%)		
amino acid	position 1	position 2	position 3
alanine	16	82	118
arginine		28	132
asparagine		41	127
aspartic acid		99	44
cysteine		77	108
glutamic acid		70	46
glutamine		70	118
glycine		32	31
histidine		60	102
isoleucine		7	61
leucine	34	53	85
lysine		27	68
methionine	84	64	119
phenylalanine	8	85	136
proline			95
serine	[100][b]	128	[100]
threonine		97	68
tryptophan		18	30
tyrosine		[100]	127
valine		22	115

[a] Activity was assayed as described in the text. Values are given in percent relative to the parent peptide (boxes). [b] On a comparative scale, S-Y-S-M-E-H-F-R gave a value of 73%.

previously (Yamada & Bradshaw, 1991). All peptides were purified by reverse-phase HPLC on a Beckman Ultrosphere ODS column (5 μm, 2.0 × 250 mm). t-Boc amino acids were from Bachem. All other synthesis reagents were obtained from ABI. Rat liver NAT was prepared by the method of Yamada and Bradshaw (1991).

Methods. NAT activity for the various substrates was determined by the method described previously (Yamada & Bradshaw, 1991) with some modifications. Reactions were initiated by the addition of 2.73 nmol of [^{14}C]acetyl-CoA and enzyme to a 1.5-mL Eppendorf tube containing potassium phosphate buffer (pH 8.0, 200 mM) and 10 nmol of the substrate peptide. The final volume was adjusted to 120 μL. Activity was determined by reverse-phase HPLC and radioactivity measurements.

Results

Effect of N-Terminal Substitutions on Substrate Specificity. The routine assay employed for the isolation of rat liver polysome NAT (Yamada & Bradshaw, 1991) used ACTH-1–8. This sequence, S-Y-S-M-E-H-F-R, is N^{α}-acetylated following postribosomal processing of the pro-opiomelanocortin precursor in the pituitary, presumably in the ER/Golgi continuum. Its modification by the cotranslationally active NAT suggests a similar specificity for the two enzymes or that the same enzyme can occur in both places. To examine the effect of amino acid substitutions in the first three positions of substrate peptides on enzyme specificity, this peptide was modified to a structure more compatible with the HPLC identification step. In this series, the first three positions were retained while the last five positions were changed to G-G-L-L-L. This sequence gave excellent retention on reverse-phase HPLC columns and allowed the facile manipulation of chain length (by inserting additional glycine residues) in studies described below.

The effect of substituting the 20 normally occurring amino acids at the N-terminus (position 1) on NAT activity is given in Table I. The activity of the S-Y-S-sequence was chosen as the reference, and the activities observed for the other peptides are given as a percentage of this value. By comparison, the ACTH-1–8 peptides showed a relative activity of 73, indicating that sequences beyond the initial three positions can exert an effect. As expected, alanine was also acetylated, although at a considerably reduced efficiency. However, no activity was found with threonine and glycine in the N-terminal positions. Although less common than acetylalanine and acetylserine, proteins with acetylthreonine and acetylglycine are regularly observed (Driessen et al., 1985). Longer sequences (see below) or sequences with different penultimate residues might show a stronger tendency to acetylate threonine or glycine in the amino-terminal position.

Somewhat more surprising was the reproducible observation that methionine, leucine, and phenylalanine were also acetylated as the amino-terminal residue of this peptide. The N^{α}-acetylation of methionine (as retained initiator methionine) has been commonly seen but generally only when the adjacent residue is aspartic or glutamic acid (or asparagine and sometimes glutamine) (Huang et al., 1987; Boissel et al., 1988). The finding that the N-terminal sequence Met-Tyr- is an excellent substrate for rat polysome NAT in vitro and that two other hydrophobic residues can also be acetylated in this sequence suggests a previous unknown activity. Several explanations are possible: (1) modifications of this type occur in situ but have not been seen before perhaps because of their rarity; (2) the specificity of the enzyme is altered in the homogeneous state, perhaps because of the absence of specifying influences of the ribosome or other proteins, such as the *ard* gene product, suggested to be a NAT subunit in yeast (Mullen et al., 1989); (3) these modifications are a peculiarity of this substrate sequence and do not normally occur with most other sequences; or (4) other activities, such as deacetylases, alter the apparent in situ specificity profile so as to obscure these modifications. Other NAT activities might also explain the recalcitrance of the N-terminal threonine and glycine peptides to modification.

Table I also indicates the observed activities of octapeptide substrates modified systematically in position 2 (with serine as the amino terminus). Relative to the same reference (S-Y-S-G-G-L-L-L), only serine in the second position (S-S-S-G-G-L-L-L) showed greater activity (128 vs 100%), indicating that tyrosine is well tolerated in this position. On the other hand, several residues depressed the activity by greater than 50%. Glycine, with no side chain, lysine and arginine, as basic residues, and valine and isoleucine, as β-branched amino acids, were distinctly inhibitory, and proline prevented N^{α}-acetylation of the serine residue completely. Tryptophan also exerted a strong negative effect. Interestingly, histidine, also a basic residue, was not as inhibiting as lysine and arginine, and threonine, another β-branched amino acid, was fully active as compared to the reference sequence. Clearly, there is no sustained pattern to these effects. The insertion of proline in the second position may be a useful tool in the expression of recombinant proteins in eukaryotic cells to block acetylation of proteins from nonsecretory constructs (Kendall et al., 1990).

The effect of systematic substitutions in position 3 of the substrate is shown in the third column of Table I. Serine and tyrosine occupied the first and second positions in all cases. Clearly, substitutions in this position exert a weaker effect, and only glycine, aspartic acid, glutamic acid, and tryptophan were notable. Considerably lesser effects were observed with threonine, lysine, and isoleucine, and several residues showed mildly enhancing effects relative to the reference peptide with serine in the third position.

Figure 1 summarizes, in a comparative fashion, the data of Table I. While the most important effector is clearly the

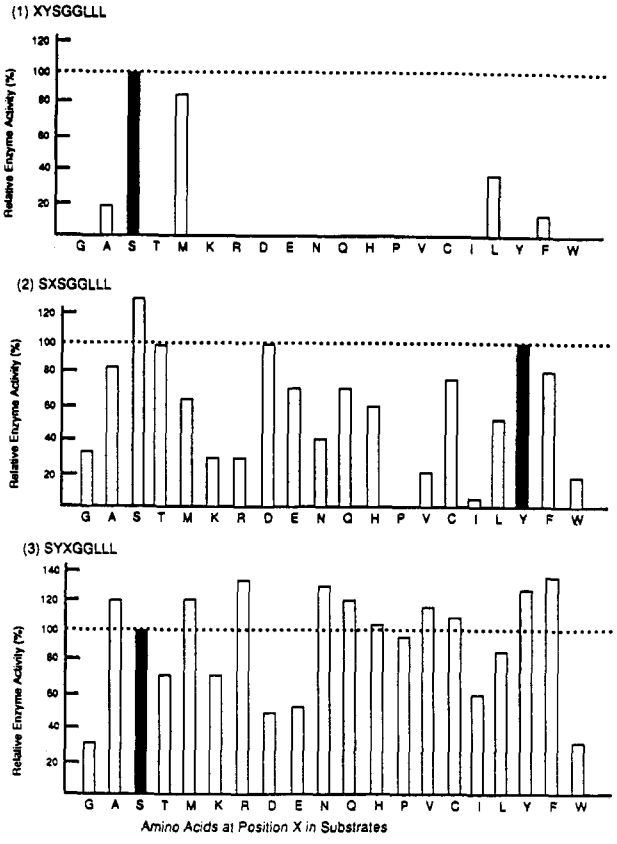

FIGURE 1: Effect of amino acid substitutions in the first three positions of the synthetic peptide S-Y-S-G-G-L-L-L on N^{α}-acetyltransferase activity. Values are given relative to the parent peptide, indicated by the solid bar in each data set.

nature of the residue in the amino-terminal position, certain amino acids in the second and third positions can clearly affect the modification. The absence of a side chain (glycine) in either position lowers the activity, as do basic residues in position 2 and acidic residues in position 3. Proline in position 2 absolutely blocks the reaction, probably because of conformational constraints, and isoleucine and valine are poorly tolerated, perhaps for similar reasons. The H-bonding capacity of threonine may overcome the restrictions otherwise imposed by the β-branching. Finally, tryptophan also appears to be inhibitory in either position. While the sum of these observations does not allow the construction, as yet, of a detailed map of the subsites in the catalytic center of the enzyme, they do provide useful guidelines for understanding the controlling elements (at least at the substrate level) of N-terminal acetylations.

Effect of Chain Length on NAT Activity. The effect of substrate chain length on NAT activity was examined with various-sized fragments of ACTH and with a series of synthetic peptides of the general structure S-Y-S-$(G)_n$-L-L-L. The results of each study are shown in Figures 2 and 3. Six peptides representing the amino-terminal sequence of ACTH (1–8, 1–10, 1–13, 1–17, 1–24, and 1–39) were assayed; the initial eight residues are identical with those of the standard substrate. Clearly, the optimal sequence length was 10 residues, and the addition of the dipeptide Trp-Gly in the C-terminal position increased activity 6-fold. Longer extensions resulted in lowered activity up to 17 residues and thereafter were generally without further effect.

The synthetic peptide series, where additional residues were added internally (as polyglycine sequences), generally showed a similar profile (Figure 3). The optimal chain length in this group was 11 residues long (a 10-residue sequence was not

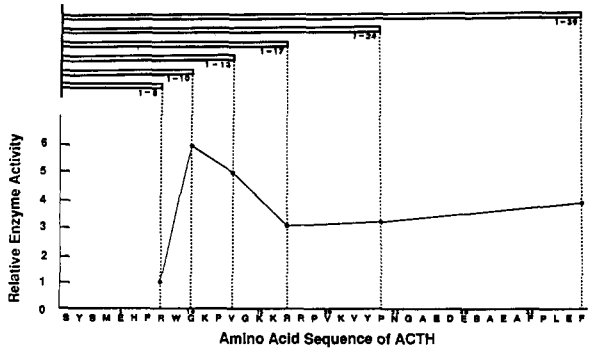

FIGURE 2: Effect of polypeptide chain length on the N^{α}-acetylation of various ACTH peptides. Activity is given as a ratio relative to des-acetyl-ACTH-1–8. Other details are given in the text.

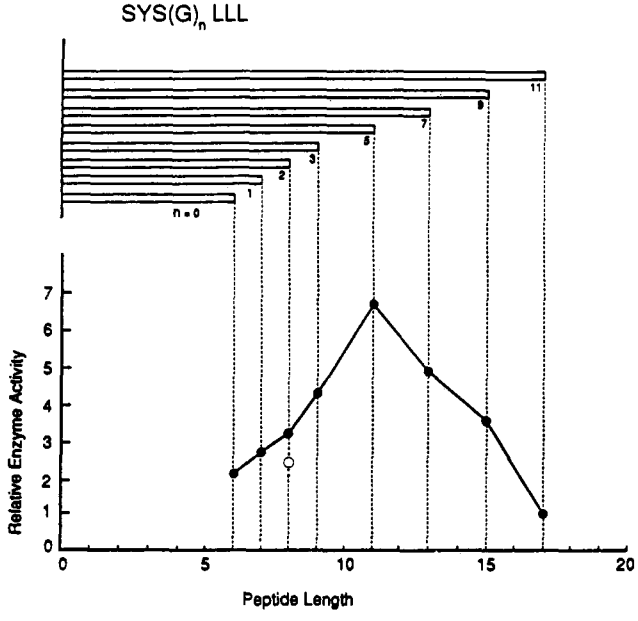

FIGURE 3: Effect of polypeptide chain length on the N^{α}-acetylation of the synthetic peptide S-Y-S-$(G)_n$-L-L-L. The length of each polyglycine insert is given at the top and the overall length of the peptide at the bottom. The open circle represents the relative activity of S-Y-S-M-E-H-F-R (des-acetyl-ACTH-1–8), the standard substrate previously employed (Yamada & Bradshaw, 1991). Activity is given as a ratio to the longest peptide (n = 11).

tested), and activity decreased linearily with longer sequences up to 17 residues (longer peptides were not tested). The relative activities of ACTH-1–8 and the synthetic peptide (n = 2; total length 8) were similar (Figure 3); however, the fold stimulation of the 10- (or 11-) residue peptides in each series was notably different. This was also reflected in the 13- and 17-residue peptides of each series (the ACTH sequences were severalfold better substrates in each case).

Discussion

The specificity of polysome rat liver NAT is affected by both the sequence and the length of its peptide substrates. In keeping with previous observations (Driessen et al., 1985; Huang et al., 1987; Boissel et al., 1988) the amino-terminal residue remains the strongest single influence. However, the spectrum of sequences presented to the enzyme, as nascent polypeptides, is influenced by the action of MAP which appears to act first. If MAP were not active, it is quite possible that a number of sequences with methionine as N-terminus might be N^{α}-acetylated that are not normally observed. The cell-free experiments of Boissel et al. (1988) are consistent with this hypothesis (see also below).

The specificity of the rat enzyme observed in vitro in these studies is not entirely that expected from the in situ experiments in yeast (Huang et al., 1987) or those found in the protein database (Bradshaw & Arfin, 1988). The lack of acetylation of N-terminal threonine and glycine residues was surprising, and the modifications of N-terminal leucine and phenylalanine were unexpected. While it is true that the former pair occur less frequently as N^{α}-acetylated N-termini, they were found to be efficiently modified in situ in yeast and in the cell-free system. The antepenultimate tyrosine does not appear to be the cause (Figure 1). It may be that a longer sequence will show greater activity with such peptides, a possibility that is currently under investigation.

The N^{α}-acetylations of the hydrophobic residues (methionine, leucine, and phenylalanine) suggest a general feature of a catalytic site of the enzyme. Neither of the last two sequences would be expected from the specificity of MAP, but N-terminal Met-Tyr sequences would be. That these have not been detected as yet in an N^{α}-acetylated form may reflect other features of this sequence or an activity of the enzyme that is affected by its environment, i.e., as detached from the ribosome. Further investigation of this activity will also be required.

Interestingly, these observed specificities for the rat liver enzyme are generally in keeping with the cell-free synthesis experiments using human globin substrates and rabbit reticulocyte or wheat germ enzymes (Boissel et al., 1988). In these experiments, a much broader range of N^{α}-acetylations was observed. Only N-terminal valine and proline of the desmethionine series and N-terminal methionine peptides followed by valine, arginine, and lysine were not modified. When valine was followed by aspartic acid, it was half-acetylated in the rabbit system. Met-Tyr was 95 and 90% modified in the rabbit and wheat germ systems, respectively. These results, which were essentially identical for the two systems (when the wheat germ system was supplemented with additional acetyl-CoA), suggest the enzyme, in a cell-free environment, shows a greater range of substrate specificity that is in part mirrored by the homogeneous enzyme. Of note is the observations of these workers that an acetyl-CoA-depleted (by a gel filtration preparation step) wheat germ system shows a specificity very similar to that seen in situ in yeast (Huang et al., 1987) and closely reflects the database observations for mature cytoplasmic proteins (Arfin & Bradshaw, 1988). This indicates that acetyl-CoA may be limiting during in situ modifications and thus contribute to the specificity of N^{α}-acetylations.

The specificity of homogeneous yeast NAT has also been examined by utilizing synthetic peptides (Lee et al., 1990b). By use of yeast alcohol dehydrogenase (1–24), whose N-terminal sequence is Ser-Ile-Pro-, peptides containing N-terminal threonine, proline, and valine were substantially modified (70–100%), and peptides with glycine, histidine, phenylalanine, tyrosine, and methionine were detectably acetylated (≤20%). Proline, which blocked modification, was the only amino acid to exert a major effect in position 2, although glycine and tryptophan were slightly inhibitory (~80% of control). These results are similar in some respects to those found for the rat liver enzyme and dramatically different in others. The lack of N^{α}-acetylation of alanine and the substantial modification of proline and valine are illustrative. Interestingly, the amino-terminal experiments utilized peptides with isoleucine in the penultimate position, which was very inhibitory to the rat liver enzyme. Studies with the hen oviduct enzyme (Kamitani et al., 1989), although limited, are basically in agreement with the specificity of the rat enzyme. Various ACTH derivatives with N-terminal serine were effectively modified but not when glycine was substituted. Insulin A chain (Gly) and Met-Lys-bradykinin were not modified.

The optimal chain length of 10–11 residues is consistent with some but not all of the observations with the hen oviduct enzyme. Kamitani et al. (1989), using ACTH derivative as well, observed no activity with peptides of five or eight residues; activity was seen with ACTH-1–10 and peaked with ACTH-1–11. However, longer chained sequences showed greater activity with ACTH-1–8, ACTH-1–24, and ACTH-1–37, being approximately 5, 10, and 9 times more active than the 11-residue peptide, respectively. In contrast, the rat liver enzyme was active with peptides as short as four residues and did not show extraordinary activities with the longer peptides. Thus the two animal enzymes are rather different in this regard.

N^{α}-Acetylation is considered to occur primarily as a cotranslational event. Since the nascent chain must be ~20 amino acids before it extrudes from the ribosome, these data would suggest that the optimal length for in situ modification would be ~30 amino acids. This is in excellent agreement with previous estimates for such activities (Palmiter et al., 1978).

Recently, Lee et al. (1990a) have described a second yeast NAT with activity for N-terminal methionine peptides but not for peptides with N-terminal alanine or serine. The enzyme is apparently selective for substrates with terminal Met-Asp, Met-Glu, and Met-Asn sequences, a specificity that agrees exactly with the in situ experiments of Huang et al. (1987). However, both the rat and yeast NATs can acetylate other substrates with N-terminal methionine. Thus, the relationship of this NAT with the NAT previously described (Lee et al., 1988) and the comparable rat enzyme described herein remains to be determined.

ACKNOWLEDGMENTS

We thank Richard Kendall, Al Stewart, Bun-ichiro Nakajima, and Stu Arfin for helpful discussions. We also thank Steve Disper and Kevin Burke of the Protein/Nucleic Acid Analysis Laboratory, UCI, for their help in the preparation of some of the substrates used in this work. Kisma Stepanich and Kathleen Sullivan provided expert assistance in the preparation of the manuscript.

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Functional Interaction of Plasminogen Activator Inhibitor Type 1 (PAI-1) and Heparin

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Received May 17, 1990; Revised Manuscript Received September 26, 1990

ABSTRACT: Plasminogen activator inhibitor type 1 (PAI-1), the fast-acting inhibitor of tissue-type plasminogen activator (t-PA) and urokinase (u-PA), is a member of the serpin superfamily of proteins. Both in plasma and in the growth substratum of cultured endothelial cells, PAI-1 is associated with its binding protein vitronectin, resulting in a stabilization of active PAI-1. Recently, it has been demonstrated that the PAI-1-binding site on vitronectin is adjacent to a heparin-binding site (Preissner et al., 1990). Furthermore, it can be deduced that the amino acid residues, proposed to mediate heparin binding in the serpins antithrombin III and heparin cofactor II, are conserved in PAI-1. Consequently, here we have investigated whether PAI-1 also interacts with heparin. At pH 7.4, PAI-1 quantitatively binds to heparin–Sepharose and can be eluted with increasing [NaCl]. Binding of PAI-1 to heparin–Sepharose can be efficiently competed with heparin in solution (IC_{50}, 7 μM). In the presence of heparin, the protease specificity of PAI-1 toward thrombin is substantially increased. This is shown by (i) quenching of thrombin activity of PAI-1 in the presence of heparin and (ii) induction of the formation of SDS-stable complexes between thrombin and PAI-1 by heparin. In a dose response curve, both effects reached a maximum at approximately 1 unit/mL and then diminished again upon further increasing the heparin concentration, strongly suggesting a template mechanism as an explanation for the observed effect. In contrast to vitronectin, heparin does not stabilize the active conformation of PAI-1. We propose that PAI-1, like antithrombin III, heparin cofactor II, and protease nexin 1, belongs to the group of heparin-dependent serpins. The binding of PAI-1 to heparin suggests that heparin may contribute to the localization of PAI-1 at particular sites, thus being involved in the regulation of plasminogen activation. Furthermore, we provide evidence that heparin has the potential to locally enhance plasminogen activation by catalyzing the thrombin-induced neutralization of PAI-1.

Serpins comprise a family of over 40 highly homologous proteins, most of which function as specific inhibitors of selected target serine proteases by forming a tight, equimolar, inactive complex (Travis & Salvesen, 1983; Huber & Carrell, 1989). The interaction between a serpin and a target protease is characterized by a fast association rate ($>10^5$ M^{-1} s^{-1}) and an extremely slow dissociation rate ($<10^{-5}$ s^{-1}). Protease specificity of a serpin is mainly determined by the amino acid sequence of the carboxyl terminally located reactive center, which functions as an exposed "bait" for the protease by mimicking a putative cleavage site (Travis & Salvesen, 1983; Carrell & Boswell, 1986).

Additional factors may contribute toward target protease specificity, since some serpins were shown to have dramatically increased association rates with certain proteases in the presence of sulfated glycosaminoglycans such as heparin. Heparin has been used for many years as a potent anticoagulant for the treatment of thromboembolic disorders in man. Its anticoagulant effect has been attributed to the acceleration of inhibition of thrombin and other proteases of the coagulation cascade by the serpin antithrombin III (Rosenberg & Damus,

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