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Studies on the specificity of acetylaminoacyl-peptide hydrolase

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Abstract

In a continuing attempt to explore the types of specificity determinants that may affect protein–protein (peptide) interactions, a number of short (2–5 residues) acetylated peptides have been compared as substrates for the enzyme acetylaminoacyl-peptide hydrolase (EC 3.4.19.1). The reference substrate was Ac-AAAA, and most of the other substrates were derived from this basic structure by single amino acid substitutions. The K_m and k_{cat} for the different substrates were determined by standard steady-state kinetics, and the corresponding $\Delta\Delta G_T^{\ddagger}$ value derived from k_{cat}/K_m was used for the comparison, setting $\Delta\Delta G_T^{\ddagger}$ for Ac-AAAA equal to 0. The best substrates were found to be those containing negative charges (Asp > Glu) or aromatic residues in positions 1′, 2′, or 3′ ($\Delta\Delta G_T^{\ddagger}$ values of 2–5 kJ); the negative charge provided by the C-terminus of the substrate also appears to be important, since the amide and *O*-Me ester derivatives caused a change in $\Delta\Delta G_T^{\ddagger}$ values of −7 to −8 kJ from the reference peptide. The stimulating effect of the negative charges is consistent with the inhibitory effect of positive charges in similar peptides (Krishna RG, Wold F, 1992, *Protein Sci 1*:582–589), and the proposed active site model incorporates subsites for both charge–charge and hydrophobic interactions. In assessing all the data, it is clear that the properties of the individual substrates reflect the total make-up of each peptide and not only the effect of a single residue in a given position. Thus, while the peptides with single Asp or Phe substitutions in 1′, 2′, and 3′ gave $\Delta\Delta G_T^{\ddagger}$ values of 3–5 kJ, the peptide containing all 3 modifications, Ac-ADDF, gave only 1 kJ. Similarly, Ac-TAAA was a poor substrate and Ac-GAAA was not cleaved at all in this study, while in the past other peptides such as Ac-TGG and Ac-GGG have been found to be excellent and reasonably good substrates, respectively. Although the rate differences observed in this work are minor, they nevertheless appear to reflect the kind of structural detail that is involved in determining the specificity of protein–protein (peptide) interactions.

Keywords: kinetic analysis; peptidase specificity determinants; protein N-terminal processing; specificity

N^{α}-acylaminoacyl-peptide hydrolase (APH; EC 3.4.19.1) catalyzes the removal of the acetylated amino acid from N^{α}-acetylated peptides in vitro. The activity has been isolated from several different mammalian sources (Witheiler & Wilson, 1972; Tsunasawa et al., 1975; Gade & Brown, 1978; Schonberger & Tschesche, 1981; Radhakrishna & Wold, 1986; Kobayashi & Smith, 1987), and the sequence has been deduced from the cloned genes of the rat liver (Kobayashi et al., 1989) and pig liver (Mitta et al., 1989) enzymes. The hydrolase has been used, with limited success, to deblock N^{α}-acetylated proteins, including the hydrolase itself, for subsequent N-terminal sequencing (Farries et al., 1991; Krishna et al., 1991; Hirano et al., 1992).

The specificity of the hydrolase is first of all determined by the acetylated amino acid to be hydrolyzed, showing strong preference for the most commonly occurring acetylated N-termini found in proteins: alanine, methionine, serine, glycine, and threonine (Witheiler & Wilson, 1972; Tsunasawa et al., 1975; Gade & Brown, 1978; Kobayashi & Smith, 1987; Radhakrishna & Wold, 1989; Yamada & Bradshaw, 1991; Krishna & Wold, 1992). However, the residues C-terminal to the scissile bond also contribute significantly to the specificity (Gade & Brown, 1978; Schonberger & Tschesche, 1981; Kobayashi & Smith, 1987; Radhakrishna & Wold, 1989; Krishna & Wold, 1992). These contributions can be summarized as follows. (1) D-Amino acids on either side of the scissile bond make the enzyme completely inactive. (2) The enzyme is most active with peptides having acetylated Ala as their N-terminus; Ac-Gly activities of 2–30% of the value for Ac-Ala have been reported for similar peptides. Acetylated Met and Ser fall in between these 2 extremes. The very limited information available for Ac-Thr is ambiguous in that one peptide shows high activity and another no activity at all. (3) Pro at either side of the scissile bond abolishes activity. Pro may have little inhibitory effect if it is the third amino acid of a tripeptide; however, if it is followed by additional amino

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acids so it no longer occupies the C-terminal position, hydrolase activity is significantly decreased. (4) As the peptide length increases, the activity of APH decreases, with N^{α}-acetylated tripeptides supporting the highest activity. (5) Positively charged amino acids (Lys, His, Arg) have a negative effect on activity, which is eliminated or diminished if the charge is neutralized, reversed, or distanced from the scissile bond. When all these reported effects are compared quantitatively, a number of discrepancies are actually observed. The lack of agreement can to some extent be ascribed to the use of different assays, but it appears that a much more compelling reason may be the facts that the structures of the substrates are subtly different, and that the discrepancies reflect the specificity of the enzyme in recognizing these structural differences.

The present work was undertaken to explore and, if possible, establish quantitative evidence that the role of a given type of residue in a given position of a short peptide may be modulated by the neighboring residues. To this end we have prepared a series of acetylated peptide substrates, determined their K_m and k_{cat} values, and compared the resulting $\Delta\Delta G_T^{\ddagger}$ values for small families of peptides, each reflecting the influence of secondary structural variables on a given primary one.

Results and discussion

The results from the present studies are reported in Table 1. The major group of peptides all had Ac-Ala N-termini, allowing the effect of the residues in the P1′, P2′, P3′ positions to be evaluated. The K_m for these substrates varied from 0.2 to 9 mM, but the majority of the peptides showed relatively minor differences in K_m in the range 0.2–3 mM. Similarly, 2 subsets of k_{cat} values were evident: $<10\ s^{-1}$ and $>20\ s^{-1}$. The variation in these 2 parameters (K_m and k_{cat}) resulted in $\Delta\Delta G_T^{\ddagger}$ values from −6.3 to +5.3 kJ, as compared to the control peptide Ac-AAAA (0 kJ). The data must be interpreted with care, because many of the relatively small variations in $\Delta\Delta G_T^{\ddagger}$ values are well within the range of possible experimental errors. However, analyzing the overall trends suggested by the K_m, k_{cat}, and $\Delta\Delta G_T^{\ddagger}$ values in Table 1 along with the data presented in previous publications allows some general conclusions to be drawn about the APH active site and the favored substrate structures. The main aspects of these conclusions are discussed below.

Table 1. *Kinetic data for cleavage of [^{14}C]acetylated peptide substrates by N^{α}-acylaminoacyl-peptide hydrolase and the associated $\Delta\Delta G_T^{\ddagger}$ (Ac-peptide) values*[a]

Substrate	K_m (mM)	k_{cat} (s^{-1})	$\Delta\Delta G_T^{\ddagger}$ (kJ)
Ac-MAAA (2)[b]	0.8	4.3	−0.6
Ac-TAAA (3)	1.5 (0.5)[c]	2.2 (0.1)	−3.9 (0.8)
Ac-SAAA (2)	3.7	2.4	−6.3
Ac-GAAA (2)	No measurable activity		
Ac-EAAA	No measurable activity		
Ac-AA (3)	0.4 (0.2)	6.8 (0.9)	2.7 (0.7)
Ac-AAA (7)	1.9 (0.7)	36.1 (15.3)	2.6 (0.5)
Ac-AAAA (7)	1.3 (0.2)	9.4 (2.2)	0.0 (0.3)
Ac-AAAAA (4)	2.7 (1.3)	9.8 (3.1)	−1.6 (0.5)
Ac-AA-OMe (2)	8.8	26.9	−2.2
Ac-AAA-OMe (3)	6.4 (0.1)	8.7 (2.2)	−4.3 (0.6)
Ac-AAA-amide	4.5	3.9	−5.4
Ac-ADAA (3)	0.7 (0.2)	27.9 (7.2)	4.3 (0.4)
Ac-AADA	0.4	20.9	5.3
Ac-AAAD	0.5	24.3	5.2
Ac-ADDF	0.2	2.7	1.2
Ac-AEAA (4)	0.2 (0.04)	3.0 (0.8)	1.7 (0.6)
Ac-AAEA	0.6	9.3	2.0
Ac-AAAE	0.2	4.3	2.6
Ac-AAAAE	1.0	5.8	−0.4
Ac-AFAA (3)	1.3 (0.5)	32.7 (9.1)	3.4 (1.4)
Ac-AAFA (3)	2.8 (0.7)	32.6 (16.9)	1.1 (0.7)
Ac-AAAF (3)	1.3 (0.8)	26.0 (6.8)	2.9 (1.7)
Ac-AAFG[d]	1.0	38.4	0.8
Ac-AARG[d]	3.3	4.1	−8.1
Ac-AAPA[d]	3.6	3.0	−9.2

[a] The K_m and k_{cat} values for the hydrolysis of the [^{14}C]acetylated peptides were determined assuming Michaelis–Menten enzyme kinetics; the $\Delta\Delta G_T^{\ddagger}$ (Ac-peptide) = $RT\ln\{[k_{cat}/K_m(\text{Ac-peptide})]/[k_{cat}/K_m(\text{Ac-AAAA})]\}$ values were calculated.

[b] Value in parentheses is the number of experiments; the average value is reported.

[c] Value in parentheses is the calculated standard error when 3 or more experiments were performed for the substrate.

[d] Values calculated from the data of Krishna and Wold (1992).

Effect of the acetylated N-terminal amino acid to be removed

In general, the hypothesis that APH is specific for the most commonly occurring N^{α}-acetylated amino acids found in eukaryotic proteins (Radhakrishna & Wold, 1989) was confirmed by the results of this study. Table 1 indicates that Ac-AAAA, which was used as the reference substrate with $\Delta\Delta G_T^{\ddagger} = 0$, was the best substrate; all the others had negative $\Delta\Delta G_T^{\ddagger}$ values. Ac-MAAA ($\Delta\Delta G_T^{\ddagger} = -0.6$) was similar to Ac-AAAA, but the Ac-Thr and Ac-Ser peptides were poorer substrates, reflected by both an equal or higher K_m value and a 4–5-fold decrease in k_{cat} when compared to Ac-AAAA. Ac-EAAA had no measurable activity. One notable difference between this and previous studies is observed for the activity of Ac-Gly-containing peptides. In this study no release of Ac-Gly from Ac-GAAA was observed. In other studies, activities were reported to be as high as 30% and as low as 2% of Ac-Ala peptide controls (Witheiler & Wilson, 1972; Tsunasawa et al., 1975; Gade & Brown, 1978; Kobayashi & Smith, 1987; Radhakrishna & Wold, 1989). Also, Radhakrishna and Wold (1989) reported that Ac-TGG was a better substrate than Ac-AGG. These discrepancies need to be examined in terms of the "context" in which the presumed specificity-determining amino acids were displayed in the different substrates. As pointed out above, the use of different assays precludes direct quantitative comparisons, and thus the different pairs of substrates will be compared in terms of rate ratios using the Ac-Ala peptides as reference. For Thr, 2 substrates have been studied: Ac-TGG/Ac-AGG = 1.8; Ac-TAAA/Ac-AAAA = 0.23 (k_{cat}). For Gly a large number of peptides can be considered: Ac-GGG/Ac-AGG = 0.3; Ac-GA/Ac-AA = 0.04; Ac-GAA/Ac-AAA = 0.1, 0, 06; Ac-GAAA/Ac-AAAA = 0 (k_{cat}); Ac-GAGGDASGE/Ac-AAGGDASGE = 0.08. It is clear that the properties of the Ac-Thr and the Ac-

Gly peptide bonds strongly reflect the composition of the rest of the peptide. A large number of similar lists of the effects of "context" variations on peptide substrates can be extracted from the literature; it is clear that it is not enough to merely state that APH requires a given residue in positions P1, P1′, P2′, etc.; the complete statement is that these residues are required in conjunction with and affected by all the other constituents of the peptide substrate. All of these variables should ideally be considered in the modeling of the active site of the enzyme.

Effect of peptide length

The effect of peptide length on APH is consistent with the data previously reported. There is an apparent discrepancy in the data for Ac-AA that actually is likely to reflect only the different analyses used. The previous data based on single substrate concentration points reported Ac-AA to be the poorest of the Ala peptide substrates. As shown in Table 1, the $\Delta\Delta G_T^{\ddagger}$ value for Ac-AA is the same as that for Ac-AAA. The k_{cat} for Ac-AA was found to be 4–5-fold less than that for Ac-AAA, and the $\Delta\Delta G_T^{\ddagger}$ values are similar because the K_m measured for Ac-AA is 5-fold lower than that of Ac-AAA (Table 1). Clearly, if the single concentration activity experiments were run at fairly high substrate concentration, the difference in k_{cat} would dominate and indicate the lower activity for Ac-AA. This experimental discrepancy needs to be considered in most of the comparisons to previous data. The longer peptides, Ac-AAAA and Ac-AAAAA, are poorer substrates mainly due to a decrease in their k_{cat} values, since their K_m values were equal to or only slightly greater than that for Ac-AAA.

Effect of negative charge

Using aspartic acid- and glutamic acid-containing peptides, and comparing C-terminally blocked peptides with their unblocked counterparts, the effect of negative charges in the peptide substrate could be evaluated. Asp at either position P1′, P2′, or P3′ of an alanine-based tetrapeptide showed a lower K_m and an increased k_{cat} when compared to Ac-AAAA (Table 1). The resulting $\Delta\Delta G_T^{\ddagger}$ values of 4.3–5.3 kJ identify these Asp-containing peptides as the best substrates of all those tested. Interestingly, the peptide Ac-ADDF, which might have been expected to be an excellent substrate for APH, was not. It again emphasizes the involvement of the entire peptide structure in determining substrate quality; although Asp in either P1′ or P2′ and Phe in P3′ (see below) all are strong positive components of the substrate peptides, the three together give a 10-fold lower k_{cat} than those observed for the individual parent peptides. Only a very low K_m makes the Ac-ADDF peptide an acceptable substrate with a $\Delta\Delta G_T^{\ddagger}$ value of 1.2 kJ.

The corresponding Glu-containing peptides, although yielding K_m values very similar to those of the Asp-containing peptides, had significantly lower k_{cat} values, resulting in $\Delta\Delta G_T^{\ddagger}$ values of 2–3 kJ. The pentapeptide Ac-AAAAE was the only Glu-containing peptide that had a negative $\Delta\Delta G_T^{\ddagger}$ value. Although the k_{cat} measured for this substrate was similar to that measured for the other Glu-containing peptides, the K_m was found to be 2–5-fold higher. This result may reflect improved enzyme–substrate interactions in the transition state for negatively charged substrates superimposed on the decreased affinity for longer peptides.

To determine to what extent the negative charge at the C-terminus of peptide substrates may also affect binding and hydrolysis of a substrate, the kinetic parameters for Ac-AA-OMe, Ac-AAA-OMe, and Ac-AAA-amide were compared to those for the unblocked substrates. As shown in Table 1, the removal of the C-terminal charge has quite substantial effects on both K_m and k_{cat}. In short, the C-terminally blocked alanine peptides were poorer substrates than their unblocked counterparts, with a decrease in $\Delta\Delta G_T^{\ddagger}$ of 5–8 kJ. The blocking group tended to mimic an additional alanine residue when comparing the k_{cat} values, while the K_m values were increased significantly in all 3 cases. There is clearly a connection between the peptide length and the C-terminal negative charge.

Based on the data presented above, we conclude that a positively charged subsite(s), which forms an ionic interaction with negatively charged substrates, is at the binding region near the catalytic site on APH. This conclusion is consistent with the data mentioned above (Krishna & Wold, 1992) showing that positively charged residues (Arg or His) are strongly inhibitory in positions P1′, P2′, and P3′ and even in positions further away from the scissile bond. The effect of pH on the inhibition by His showed unequivocally that it is the positive charge and not the bulky imidazole ring that causes the inhibition. Most of the studies of the effect of positive charges involved single concentration data points; only 1 case of an Arg-containing peptide subjected to kinetic analysis is available and is included in Table 1.

In an attempt to further explore the nature of the charge effects observed with the negatively charged substrates, the kinetic parameters for Ac-AAAA, Ac-ADAA, and Ac-ADDF were redetermined in the presence of 0.5 M NaCl (data not shown). The effects of high salt were not very dramatic; for all 3 substrates the k_{cat} was reduced about 50%, and the K_m for Ac-AAAA and Ac-ADDF was essentially unaffected. For Ac-ADAA the K_m increased about 3-fold, to give a significant decrease of 4.4 kJ in $\Delta\Delta G_T^{\ddagger}$. This observation may support the proposed involvement of charge–charge interactions in substrate binding, but the anomalous behavior of the Ac-ADDF substrate remains enigmatic.

Effect of a hydrophobic side chain

It might be expected that if an enzyme favors negatively charged side chains for binding and hydrolysis of a peptide substrate, peptides with hydrophobic side chains would be poor substrates. However, this is not the case with APH. The series of tetrapeptides that contained Phe at positions P1′, P2′, and P3′ gave K_m and k_{cat} values similar to those for Ac-AAA, one of the better substrates for the enzyme (Table 1). The increase in $\Delta\Delta G_T^{\ddagger}$ is due primarily to a significant increase in k_{cat}. In this connection it is of interest to reconsider in a little more detail the His effects that were discussed above. Performing the standard assay with His-containing peptides between pH 5.0 and 8.7 demonstrated that the positively charged side chain (Ac-AH$^+$A and Ac-AAH$^+$A) supported no activity, whereas the neutralized His-containing peptides (Ac-AHA and Ac-AAHA) containing the aromatic side chain in fact showed considerably more activity than did the Ac-AAA or Ac-AAAA controls (Krishna & Wold, 1992). In designing the active site for the enzyme, it thus appears that both an ionic interaction subsite and a hydrophobic interaction subsite need to be considered.

A view of peptide specificity determinants and a model of the active site

The maturation of preproteins into their final active structures may include several co- and posttranslational steps, involving both peptide bond cleavages and chemical modifications at the N-terminus, the C-terminus, and amino acid side chains. The specificity of all of these reactions is truly remarkable in selecting a single or only a few peptide bonds or amino acid residues for the modification reactions, and a good deal of effort is directed toward understanding the structural bases for such specificity. Ultimately they will have to be described in terms of all the amino acid sequences that interact in determining the folding, the covalent modification, the processing pathways, and the compartment location of all proteins. In trying to define the uniqueness of a given short peptide sequence it is becoming clear that even a common covalent structure may be made different from all the other like structures by the particular environment in which it is displayed. Thus, it seems reasonable to propose that the amino acid residues or short sequences involved as substrates in highly specific reactions always must be "read" in the proper "context" in order to have their unique character recognized. The context is the total complicated 3D matrix in which the substrate component exists; it includes the components of the immediate linear covalent structure as well as the broader environment created by the secondary, tertiary, and quaternary interactions in the folded structure. To understand the remarkable specificity of co- and posttranslational reactions, both the short-range direct sequence effects and the long-range 3D components of the context need to be understood. As a processing enzyme working on short peptide substrates, APH was selected as a valid probe of the direct sequence effects.

A model of the APH active site is presented in Figure 1 to summarize the information available at this time, including both the conclusions from the K_m, k_{cat}, and $\Delta\Delta G_{T^\ddagger}$ values presented in this report and data presented in previous publications. The model is based upon computer-generated 3D peptide model substrates bound at a hypothetical active site essentially copied from that derived for γ-chymotrypsin (Brady et al., 1990a) and compared for a number of Ser esterases (Brady et al., 1990b). There is clear evidence that APH is a serine protease, so the catalytic triad of Ser-His-Asp is appropriate for the APH model. The main subsites included are those accommodating the substrate's negative charges and hydrophobic side chains. The details are discussed in the legend to the figure and in the text to the accompanying Kinemage 1. The actual active site will clearly be much more complicated, especially when one attempts to incorporate the subtle interactions between subsites involved in reading the substrate sequence in the proper context. Although an active site construction such as this one may not give a precise, realistic picture of the enzyme of interest, it does, like all scientific models should, provide a valuable basis for further experiments.

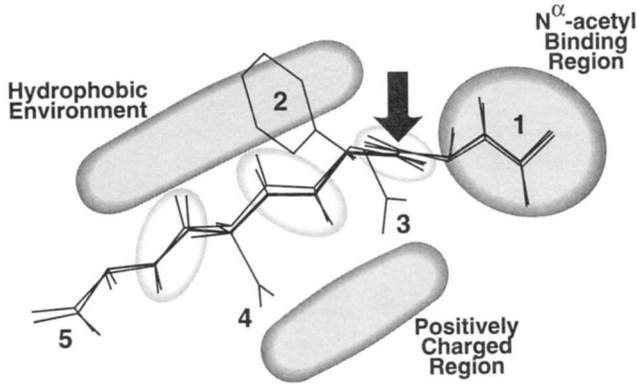

Fig. 1. Proposed model of the APH active site. Three "good" peptide substrates (Ac-AAA, Ac-ADAA, Ac-AFAA) for hydrolysis by APH were first superimposed onto a hypothetical enzyme surface using computer modeling; the salient features of the active site were then added using the published properties of chymotrypsin (Brady et al., 1990a) as the basic model. The numbered features refer to: (1) N^{α}-Ac-Ala of the peptides bound at the N^{α}-acetylated amino acid binding site of APH. This portion of the binding site is believed to dominate the specificity determinants of recognition, the order of recognition being Ac-Ala > Ac-Met > Ac-Thr/Ac-Ser > Ac-Gly. (2) The side chain phenyl group of Ac-AFAA oriented in the proposed hydrophobic environment of APH. The hydrophobic interaction between substrate and the hydrophobic environment is thought to stabilize a productive conformation of the peptide backbone, positioning the scissile bond for hydrolysis. (3) The side chain carboxyl of Ac-ADAA, and (4) the C-terminal carboxyl of Ac-AAA, both in an orientation that would maximize the hypothesized ionic interaction with the positively charged region of APH. The ionic interaction is believed to anchor the peptide in an orientation that would position the scissile bond for efficient catalysis, as well as provide a general attraction for substrate binding. (5) The C-terminal carboxyl of Ac-ADAA and Ac-AFAA. The C-terminal carboxyl of peptides longer than 3 amino acids is believed to be too far removed from the positively charged region to significantly influence the formation of a productive peptide orientation necessary for hydrolysis, but may be necessary for initial substrate binding via a general ionic attraction to the active site.

Conclusions

The goal of these studies was to evaluate the effect of the nature of the amino acid residues in neighboring positions on the expression of a given residue in a short peptide substrate. Extensive studies of other enzymes acting on peptide and protein substrates have been carried out in the past, elucidating both the essential substrate binding components causing several-order-of-magnitude changes in k_{cat}/K_m (e.g., Scarborough et al., 1993) and evaluating the more subtle effects (k_{cat}/K_m variations <10) of variations in neighboring residues (e.g., Griffiths et al., 1992). The results clearly show that it is not enough to identify a given residue in, for example, position P2′ as having a positive or negative effect on the substrate quality, since that effect is always modulated by the residue in P1′ and/or P3′. Although the effects are relatively minor for the short peptides with essentially total conformational freedom used in this study, the detailed kinetic analysis makes it possible to identify the differences as significant reflections of the total peptide structure. It would seem that these subtle differences are valid illustrations of 1 type of structure modulation that can make a given short sequence in a protein/peptide a truly unique structure as a substrate for processing enzymes or as a component in any other protein/peptide–protein/peptide interaction.

Materials and methods

Materials

APH was purified from young rabbit muscle (Pel-Freez) according to Radhakrishna and Wold (1989), and the purity was con-

firmed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). The following peptides were purchased from Bachem Bioscience Inc.: AA, AAA, AAAA, AAAAA, AA-OMe, AAA-OMe, AAA-amide. *t*-Butyloxycarbonyl- and benzyl ester- and ether-protected amino acids were obtained from Advanced Chemtech. [^{14}C]Acetic anhydride was from New England Nuclear Research Products, DuPont. All other chemicals used were reagent grade.

Synthesis, purification, and characterization of peptides

Peptides that were not purchased were synthesized by commonly used solution-phase peptide synthesis procedures. In general, the synthesis of peptides containing γ-*O*-benzyl-glutamic acid, β-*O*-benzyl-aspartic acid, *O*-benzyl-threonine, *O*-benzyl-serine, phenylalanine, glycine, and methionine was accomplished by the activation of the C-terminus of an amino acid (or peptide) to the *N*-hydroxysuccinimide ester, which was then coupled to the next amino acid in carbonate buffer according to prescribed procedures (Bodanszky, 1988). Intermediate product isolation was by extraction with ethyl acetate before and after acidification of the reaction mixture. Removal of the *t*-butyloxycarbonyl protecting group was accomplished by incubating the crude peptide in trifluoroacetic acid for 30 min at room temperature. The trifluoroacetic acid was removed in a nitrogen stream for 30 min, after which water was added and the solution lyophilized overnight. The benzyl protecting group was removed by palladium-catalyzed hydrogenation in methanol. In the case of the phenylalanine-, glycine-, and methionine-containing peptides, only the *t*-butyloxycarbonyl protecting group was present, so the trifluoroacetic acid deprotection step was the only step necessary to liberate the final peptide.

Purification of the final products was by low-pressure reverse-phase chromatography using a LoBar LiChroprep RP-18 column (3 × 30 cm, 40–63 μm; Merck). Each peptide was loaded and run isocratically with a solution of methanol/water with 0.1% trifluoroacetic acid. The exact percentage of methanol used for each purification was determined as that which optimized separation of the peptide product from contaminants based on HPLC using a HiBar LiChrosphere C-18 column (Merck).

Peptide purity was confirmed by proton-NMR using a General Electric QE300 machine, amino acid analysis performed with the LKB Alpha Acid analyzer, and in some instances, N-terminal amino acid sequencing on an Applied Biosystems 477A gas phase protein sequencer.

Preparation of substrates

Peptide acetylation was performed directly with [^{14}C]acetic anhydride using a procedure adapted from Radhakrishna and Wold (1990). In general, 50 μmol of a given peptide was acetylated with [^{14}C]acetic anhydride (5 μCi = 185 Bq, 2.6 × 10^{-4} mol) in 1 M sodium carbonate, pH ~10. Following lyophilization, the acetylated peptide was resuspended in 1–2 mL of 0.1% trifluoroacetic acid, filtered, and purified by reverse-phase HPLC using an Alltech C18 column (250 × 4.6 mm, 5 μm) eluted with an empirically selected, optimal linear acetonitrile gradient. The [^{14}C]acetylated peptide peaks from several runs were pooled, lyophilized to dryness, then resuspended in 1 mL of 50 mM NaP_i, pH 7.0, with 1 mM EDTA and 2 mM $MgCl_2$. The solution was filtered and the final specific activity determined by counting with an LKB 1209 Rackbeta liquid scintillation counter and acid hydrolysis followed by amino acid analysis with the LKB Alpha Acid analyzer. To ensure that the peptides [^{14}C]Ac-SAAA and [^{14}C]Ac-TAAA were not doubly acetylated at the N-terminus as well as the side chain, fast-atom bombardment mass spectroscopy was performed with a Kratos MS-50RF double focusing mass spectrometer fitted with an Ion-Tech saddle-field neutral atom gun operated at 8 kV (data not shown).

APH activity assay and kinetic calculations

The enzyme assays followed the general procedure of Radhakrishna and Wold (1989). A typical reaction was incubated at 37 °C and contained 0.025–0.10 μg of APH and 0.10–8.0 mM [^{14}C]acetylpeptide substrate in 50–200 μL of assay buffer (50 mM NaP_i, pH 7.0, 1 mM EDTA, 2 mM $MgCl_2$). Reaction times varied from 10 to 120 min, depending upon the substrate. The reaction was stopped by heating for 5 min at 100 °C or by quick-freezing in liquid N_2. The radiolabeled product, in most cases [^{14}C]Ac-Ala, was separated from the remaining substrate by reverse-phase HPLC using either an Alltech C18 column (250 × 4.6 mm, 5 μm) or a Beckman Ultrasphere PTH column (250 × 4.6 mm). Fractions were collected (0.25–1.0 min), scintillation cocktail added (Econo-Safe, Research Products International), and the ^{14}C dpm determined using an LKB Rackbeta liquid scintillation counter.

Assuming that APH follows Michaelis–Menten kinetics, the K_m and V_{max} of a given peptide substrate were determined by plotting a double reciprocal graph using the velocity and substrate concentration. The velocities were calculated from the percentage of product ([^{14}C]Ac-Ala) released, the value never exceeding 15% of the initial substrate concentration. The k_{cat} ($V_{max}/[E]_0$) was calculated from the measured V_{max}. The preference of APH for different substrates was evaluated by comparing their k_{cat}/K_m values. These relative rates were expressed as differences in $\Delta G_T^{\ddagger}$ values, $\Delta\Delta G_T^{\ddagger}$. For each acetylpeptide, the free energy of transition state binding was compared to Ac-AAAA:

$$\Delta\Delta G_T^{\ddagger}(\text{Ac-peptide}) = \Delta G_T^{\ddagger}(\text{Ac-AAAA}) - \Delta G_T^{\ddagger}(\text{Ac-peptide}) = RT \ln \frac{k_{cat}/K_m(\text{Ac-peptide})}{k_{cat}/K_m(\text{Ac-AAAA})}.$$

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