Stereochmical Aspects of Amine Substrate Attachment to Acyl Intermediates of Transglutaminases

HUMAN BLOOD PLASMA ENZYME (ACTIVATED COAGULATION FACTOR XIII) AND GUINEA PIG LIVER ENZYME

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Some preliminary proposals as to the mode of amine substrate attachment to acyl intermediates of guinea pig liver transglutaminase and human blood coagulation factor XIIIa were formulated from substrate and inhibitor studies (Gross, M., Whetzel, N. K., and Folk, J. E. (1977) J. Biol. Chem. 252, 3752-3759). The present study supplies more concrete explanations for the stereopreference toward peptide-bound L-lysine residues and for the increase in specificity resulting from the nonpolar side chain of an L-amino acid residue on the amino side of, and directly adjacent to, the substrate lysine residue. The amines tested were: (a) peptide derivatives of the basic structure R1CH2ONHCH2R2, R2CH2SO2-NHCH2R2, and R1CH2NHCOCH2R2, where R1 is any hydrophilic group of the lysine residue. Stereopreference resides in the proper orientation of the α-hydrogen atom and the side chain of the L-lysine residue with respect to the enzyme surface. There is a binding site on the enzyme that exists in the form of a crevice or pocket of fixed dimensions into which the side chain of a lysine residue is injected. These sites become more capacious as a function of the distance from this region. The amines are arranged in these sites with their methylene side chains in a transglutaminases catalyze a calcium-dependent acyl transfer reaction in which γ-carboxamidine groups of peptide-bound glutamine residues are the acyl donors and primary amine groups in a variety of compounds may function as acyl acceptors (for review, see Ref. 1). This reaction yields mono- or di-substituted γ-amides of peptide-bound glutamic acid. Participation of α-amine groups of peptide-bound lysine residues as acyl acceptors gives rise to ε-(γ-glutamyl)lysine cross-links (for review, see Refs. 1 and 2).

The findings reported in an earlier communication from this laboratory (3) allowed some speculation on the manner in which amine substrates attach to the acyl-enzyme intermediates of transglutaminases. These findings also supplied evidence for similar amine binding modes in the guinea pig liver enzyme and in human blood plasma factor XIIIa (activated coagulation factor XIII). The findings are, in brief, as follows. (a) Specificity for aliphatic isobranched chain primary amines is directly related to the length of their carbon chains. The greatest preference is for a branched chain amine that possesses a methylene chain equal in length to that of the side chain of a lysine residue. (b) α-Hippuryl-L-lysine amide (benzoylglycyl-L-lysine amide) is a significantly better substrate than α-hippuryl-L-lysine amide. (c) Straight chain aliphatic amines display substrate properties similar to that of the heptapeptide derivative, Nα-acetyl-Gly+-Lys-Gly3. (d) Substitution of an L-leucine residue for a glycine residue in the heptapeptide derivative, Nα-acetyl-Gly3-Lys-Gly3, does not change specificity, except in those cases where leucine is directly adjacent to lysine. (e) The heptapeptide derivative containing the Leu-Lys sequence is a substantially more specific substrate; that with the Lys-Leu sequence appears to be a less specific substrate.

Based on these observations, it was proposed that the amine attachment sites in acyl intermediates of transglutaminases are spatially restricted close to the region in which the charged amino group is positioned for reaction with the acyl-enzyme thiol ester bond. These sites become more capacious as a function of the distance from this region. The amines are arranged in these sites with their methylene side chains in a fully (or almost fully) extended conformation. The dimensions and conformations of the amine binding sites appear to be fashioned for accommodation of the side chains of poly-peptide-bound lysine residues. This is in accordance with the
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Several observations, even when taken together, did not supply a substantial enough base for further proposals concerning the amine sites of the acyl-enzyme intermediates. Among these were the apparent preference for L-lysine over the D isomer in the hippuryl amide compounds, the seemingly equivalent substrate properties of straight chain alphatic amines and of several L-lysine-containing polypeptides, and the effects observed with the heptapeptide derivatives in which an L-leucine residue is directly adjacent to the L-lysine residue. The results presented in the present communication serve to clarify and extend these earlier observations. They clearly define a stereospecificity for peptide-bound lysine residues manifested as a strong preference for the L isomer, and provide explanations for this specificity and for the manner in which the hydrophobic side chain of an L-amino acid directly adjacent to lysine in the peptide influences specificity. In addition, the data presented here supply further evidence for the very similar amine binding modes in the acyl-enzyme intermediates of the liver and blood transglutaminases.

A number of primary amines have been designed and tested as inhibitors of fibrin cross-linking by factor XIIIa (for review, see Refs. 4 and 5 and their bibliographies). Some of these amine inhibitors are similar, but not identical, in structure with several of the compounds tested here. The results obtained and the conclusions drawn from these fibrin cross-linking inhibition studies, however, do not directly relate to those reported here.

EXPERIMENTAL PROCEDURES

A previous publication in this series (3) describes in detail the enzymes, certain other materials, and the preparation of solutions used in this work. The general procedures for the synthesis of the N\(^{-}\)acetyllysine-containing heptapeptides are given in the miniprint supplement to the preceding paper (3). The supplement at the end of this paper presents pertinent analytical data on, and certain properties of additional N\(^{-}\)acetyllysine-containing heptapeptides used in this study, as well as details of the synthesis of, and analytical data on, the D and L forms of N\(^{-}\)acetylglycine and their derivatives, the \(\alpha\)-hydroxy-\(\epsilon\)-aminocaproic acids and methyl esters, the N\(^{-}\)ethyl-\(\epsilon\)-aminocaproamides, the \(\epsilon\)-aminoacylglutamides, and the \(\epsilon\)-aminoacylkylnitroso compounds. Also presented in this supplement (in Part 2) are the equations used, the definitions of constants, a brief description of the kinetic mechanism for transglutaminases, and the method of data fitting, together with the incubation conditions and methods of assay.

RESULTS

Figs. 1 through 3 present, in graphic form, the inhibitor constants, \(K_a\) values, for the amines obtained by fits of data collected from rate studies using the acetylated B chain of oxidized insulin in which the e-amino group is blocked. In the cases of lysine, or 1 lysine residue and 1 leucine residue which are adjacent to one another. With both enzymes, the peptides containing L-lysine give higher \(-\log K_a\) values than their D-lysine analogs, with the single exception in the case where D-leucine is located on the amino side of lysine.

In Fig. 2 are plotted constants obtained with labeled and D-lysine residues in a series of heptapeptide derivatives containing L-lysine or D-leucine, or 1 lysine residue and 1 leucine residue which are adjacent to one another. With both enzymes, the peptides containing L-lysine give higher \(-\log K_a\) values than their D-lysine analogs, with the single exception in the case where D-leucine is located on the amino side of lysine.

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\(1,2\) This material (including Tables 1 to IV and additional references) is published as part of a miniprint supplement immediately following this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9500 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-1075, cite author(s), and include a check or money order for $1.65 per set of photocopies.

\(3\) The material is presented in Part 2 of a miniprint supplement immediately following this paper. See Footnote 1 for further details.
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FIG. 2. Relationships of the structure and stereochemistry of lysine, lysine derivatives, and lysine analogs to the $K_a$ values obtained with these compounds as inhibitors of amine incorporation by liver transglutaminase (A) and factor XIIIa (B). Data of Table IV. The form of lysine is given above the bars. Explanation for the forms in quotations for α-hydroxy-α-aminocaproic acid and its methyl ester is given in the miniprint section.∗

and carboxyl-blocked lysine derivatives, a preference for the L form of lysine is observed.

The data plotted in Fig. 3 are for a variety of compounds, each containing a single primary amino group and a single amide bond (carboxamides and sulfonamides). In the mono-

liver transglutaminase. They found that amino acids, other than lysine, did not serve as substrates, whereas several amino acid amides were substrates. There was an earlier report by Schweet (9) that a calcium-dependent lysine-incorporating system from guinea pig liver was specific for the L form of lysine, that this amino acid was incorporated exclusively through its ε-amino group, and that other amino acids were not incorporated. The experimental conditions used in the two studies were somewhat different. However, it seems quite likely that the enzymic activity observed by Schweet (9) was that of transglutaminase. On the basis of these observations, one may conclude that only the ε-amino group of free lysine functions as a substrate for the transglutaminases. Lysine amide and esters were not tested here because of the possible participation of their α-amino groups as substrates.

acylamides of cadaverine (Group I, Fig. 3), the primary amino group is separated from the nitrogen of the amide bond by the same number of carbon atoms as is the ε-amino group of a lysine residue from a peptide bond involving its α-amino group. The sulfonamides of Group II (Fig. 3) are analogs of the carboxamides of Group I. In the Group III compounds of Fig. 3, the ε-aminocaproylamides, the number of carbon atoms between the primary amino group and the carbonyl of the amide bond is the same as that between the ε-amino group of a lysine residue and a peptide bond involving its carboxyl group. The radical, R, of the compounds of Fig. 3 is identical with the side chains of various amino acids. Thus, one of the amides of Group I (R = (CH₂₃)₃CHCH₃) is analogous to a leucyl-lysyl sequence; one of the Group III amides (R = (CH₂₃)₃CHCH₃) is analogous to a lysyl-leucyl sequence. None of the compounds, however, contain asymmetric carbon atoms. The −log $K_a$ values for most of the Group I compounds and for those in Group II compare favorably with that for the
**Figure 3.** Inhibition of transglutaminase-catalyzed amine incorporation by compounds that are analogs of X-Lys sequences (I and II) and Lys-X sequences (III) (where X is an amino acid residue with a nonpolar side chain); influences of the acyl and alkyl groups and the sulfonamide group. A, liver enzyme; B, factor XIIIa. Data of Table IV.

**Discussion**

The $K_{is}$ values for amines obtained from inhibition of methyamine incorporation into the acetylated B chain of oxidized insulin with either liver transglutaminase or factor XIIIa (Table IV) may be taken as relative measures of specificity of the acyl-enzyme intermediate of the given enzyme for these amines (3). Greater specificity is reflected in the higher $-\log K_{is}$ values presented in the figures.

Comparison of the constants obtained for the isomeric forms of $N^\alpha$-acetyl-Gly$_3$-Lys-Gly$_3$ (Fig. 1) reveals a preference of the acyl-enzyme intermediates for L-lysine over D-lysine in a simple polypeptide. This stereopreference also holds for free lysine$^2$ and for $N^\alpha$-acetyllysine, as well as for simple derivatives of lysine in which the $\alpha$-amino group and carboxyl group are blocked by acetyl and amide or methyl ester, respectively (Fig. 2). Clearly, the charged carboxyl group of lysine and of $N^\alpha$-acetyllysine reduces specificity as is evident upon comparison of $\epsilon$-aminocaproic acid and its methyl ester, and the two forms of $\alpha$-hydroxy-$\epsilon$-aminocaproic acid and their methyl esters. The fact that the two forms ("I," that originating from L-lysine, and "D," that originating from D-lysine) of $\alpha$-hydroxy-$\epsilon$-aminocaproic acid show very similar $-\log K_{is}$ values, and that the same is true of their methyl esters, suggests a dominant role in the stereospecificity of the peptide bond involving the $\alpha$-amino group of lysine.

$\epsilon$-Aminocaproic acid methyl ester, the methyl esters of the two forms of $\alpha$-hydroxy-$\epsilon$-aminocaproic acid, the L forms of $N^\alpha$-acetyllsine amide and methyl ester, and the aliphatic amines, n-hexylamine and d-methyl-n-hexylamine (all in Fig. 2), and $N^\alpha$-acetyl-Gly$_3$-Lys-Gly$_3$ (Fig. 1) all display similar $-\log K_{is}$ values. The similarity in specificity toward these compounds, the structures of which vary considerably, would
seem to rule out strong binding to the enzyme as responsible for the observed stereospecificity toward lysine residues. Rather, one would be inclined to conclude that the preference for L-lysine residues occurs as a consequence of an unobstructed fit on the enzyme surface of groups attached to the α carbon atom of L-lysine. Parenthetically, the lower specificity for D-lysine residues would derive from obstacles on the enzyme surface to the most productive arrangement of groups attached to the asymmetric carbon atom of D-lysine.

A preference for the L-lysine residue over the D-lysine residue is apparent in each of the Nα-acetyl heptapeptides of Fig. 1, with the exception of those having the D-Leu-Lys sequence. Earlier studies (3) showed that, of the Nα-acetyl heptapeptides containing L-α-amino acids, only those two in which leucine was directly adjacent to lysine displayed constants significantly different from that found for Nα-acetyl-Gly-L-Lys-Gly. The high specificity for the peptide derivative containing the L-Leu-L-Lys sequence, the lowest specificity observed for that with the L-Leu-D-Lys sequence, and the apparent inconsistency in the findings with the D-Leu-Lys-containing peptide derivatives can all best be explained by assuming a specifically oriented binding site on the enzyme surface for the hydrophobic side chain of an L-leucine residue (and possibly for those of other amino acid residues). Attraction through the hydrophobic interaction of this side chain with the enzyme enhances specificity for the L-lysine residue by increasing the frequency of productive orientations of the side chain of the L-lysine residue. The low specificity for the peptide derivative that contains the L-Leu-D-Lys sequence is the result of a weakened specificity for the D-lysine residue. This results because the force of binding of the hydrophobic side chain of the L-leucine residue is strong enough to divert the polar side chain of the D-lysine residue from a productive conformation.

In the event that the side chain of a D-leucine residue binds at the position on the enzyme surface that is designed for binding of L-amino acid side chains, a change would be expected in the alignment of adjacent amino acid residues having hydrophilic side chains. Thus, the low in preference for the L-lysine residue in the peptide derivatives that have the D-Leu-Lys sequence (Fig. 1) probably results from distortions conferred through binding of the hydrophobic side chain of D-leucine at that site on the enzyme intended for binding of L-amino acid side chains.

An L-leucine residue on the carboxyl side of, and directly adjacent to, a lysine residue appears to cause some loss in specificity for the L-lysine residue (compare results with the peptide derivatives containing Gly-Lys-Gly and Gly-Lys-L-Leu-L-Leu sequences, Fig. 1). However, since the effect is not pronounced, it seems doubtful that this change is due to specific binding of the side chain of the leucine residue. A more likely explanation is that the bulky side chain of leucine interferes with the proper association through some degree of steric hindrance. This contention is supported by the observation that a D-leucine residue on the carboxyl side of, and adjacent to, the lysine residue has essentially no effect on specificity.

An alternative explanation for the effect on specificity of the L-leucine residue on the carboxyl side of L-lysine was offered earlier (3) and was presented as a basis for further experimentation. It was suggested that the lower specificity could originate from a reverse alignment of the L-Leu-L-Leu-containing peptide derivative along the enzyme surface. This would lead to an arrangement of the lysine residue with its α-hydrogen atom in a reversed position with reference to the enzyme surface. Implied was the concept that this peptide derivative could be endowed with optimal, or near optimal, specificity by simply rearranging the groupings attached to the asymmetric carbon atoms of its lysine and leucine residues, i.e. by changing the optical configuration of the two optically active amino acid residues. The plots given in Fig. 4 contradict this notion. These replots of some of the data from Fig. 1 facilitate comparison of specificities toward peptide derivatives, the leucine and lysine α-hydrogen atoms and side chains of which can assume similar spatial orientations upon arrangement of their polypeptide chains in opposite directions. Clearly, there is little similarity in the -log $K_a$ values for the peptide derivatives in most of these pairs and there is no consistency in differences between these values in the various pairs of peptide derivatives. The concept that preference for L-lysine derives from a single directional alignment on the enzyme surface of the substrate portion of the peptide chain is supported by these comparisons.

Evidence is presented in Fig. 3 that this alignment of substrate on the enzyme surface is directed by the correct fit of the peptide bond, or some part of the peptide bond, formed through the α-amino group of the substrate lysine residue. Here, the compounds of Group I are analogs of X-Lys sequences, where X represents an amino acid residue containing a nonpolar side chain. The magnitude of the -log $K_a$ values obtained with these compounds denotes high specificity and suggests that other amino acids adjacent to lysine may exert effects similar to that observed with leucine. The compounds of group III which are analogs of Lys-X sequences show significantly lower specificities as reflected in their lower -log $K_a$ values. Arrangement on the enzyme surface of the non-

![Fig. 4](http://www.jbc.org/) Comparison of the $K_a$ values obtained for the Nα-acetyl heptapeptides that attain similar spatial orientations for the side chains and α-hydrogen atoms of lysine and leucine residues upon arrangement of their polypeptide chains in opposite directions. A replot of certain data of Fig. 1. A, liver enzyme; B, factor XIIIa. The forms of the optically active amino acid residues are given above the bars and refer, respectively, to the residues in the sequences shown.
polar group and the amino group in each of these compounds is not restricted by asymmetry around carbon atoms that correspond in position to the α carbon atoms of optically active amino acid residues. Therefore, one may conclude that the amide bond in each of these compounds can be aligned in only a single direction along the enzyme surface.

The compounds of Group II in Fig. 3 are analogs of those of Group I. In the amide portion of these compounds, the carbonyl group has been replaced by a sulfonyl group, a significantly bulkier group, but one with bond angle and bond length similar to those of the carbonyl group. It is obvious from a comparison of -log Ks values that no loss in specificity occurs as a result of this change in structure. Thus, the positioning on the enzyme surface necessary for proper orientation of both the hydrophobic group and the reactive amine does not appear to be significantly altered by a substantial increase in size of one part of the amide group. On the basis of this finding, it seems likely that the single directional alignment of the amide bond along the enzyme surface is directed by some feature of enzyme topography that accommodates the NH size of one part of the amide group. Therefore, one may conclude that the amide bond in the enzyme surface may be significantly bulkier group, but one with bond angle and bond length similar to those of the carbonyl group. It is obvious from a comparison of -log Ks values that no loss in specificity occurs as a result of this change in structure. Thus, the positioning on the enzyme surface necessary for proper orientation of both the hydrophobic group and the reactive amine does not appear to be significantly altered by a substantial increase in size of one part of the amide group.

It was proposed that the site for amine side chain attachment is spatially restricted close to the region in which the uncharged amino group is positioned for reaction, but is more capacious as a function of the distance from this position (3). It is conceivable that this site is in the form of a crevice or pocket of fixed dimensions predicated to some degree by the structure of the acyl portion of first substrate. Since a conformational change must occur in order for the last product, the γ-glutamyl product, to be released, it does not seem imperative that alterations in conformation of the acyl-enzyme intermediates occur in order for these intermediates to express their specificity toward amine substrates.

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REFERENCES


Additional references are found on pp. 660-661.
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division 1

FUNCTION AND TISSUE DISTRIBUTION OF TRANSGLUTAMINASES

There is substantial evidence that both the tetrahedral and the transfer reactions involved in the transglutaminase-catalyzed reaction proceed through acyl-enzyme intermediates formed between the catalytic triad residues of the \(\alpha\)-helix domain and the acyl group of the amine substrate. In the case of the tetrahedral intermediate, the amine substrate is covalently linked to the enzyme through a lysine residue, and in the case of the transfer reaction, the amine substrate is covalently linked to the enzyme through a carboxylic acid residue.

**Scheme 1**

With respect to the tetrahedral intermediate, the amine substrate is covalently linked to the enzyme through a lysine residue, and in the case of the transfer reaction, the amine substrate is covalently linked to the enzyme through a carboxylic acid residue.

**Equation 1**

\[
\frac{d[D]}{dt} = k_1 [A] [D] - k_{-1} [A][D] + k_{2} [B][D]
\]

This equation describes the rate of formation of the tetrahedral intermediate (\([D]\)) in the presence of the amine substrate (\([A]\)) and the enzyme (\([B]\)).

**Equation 2**

\[
\frac{d[D]}{dt} = k_1 [A] [D] - k_{-1} [A][D] + k_{2} [B][D]
\]

This equation describes the rate of formation of the tetrahedral intermediate (\([D]\)) in the presence of the amine substrate (\([A]\)) and the enzyme (\([B]\)).

**Equation 3**

\[
\frac{d[D]}{dt} = k_1 [A] [D] - k_{-1} [A][D] + k_{2} [B][D]
\]

This equation describes the rate of formation of the tetrahedral intermediate (\([D]\)) in the presence of the amine substrate (\([A]\)) and the enzyme (\([B]\)).

**References**

Stereochemical aspects of amine substrate attachment to acyl intermediates of transglutaminases. Human blood plasma enzyme (activated coagulation factor XIII) and guinea pig liver enzyme.
J Schrobe and J E Folk