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Subsites of Trypsin Active Site Favor Catalysis or Substrate Binding

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Enzymes enhance chemical reaction rates by lowering the activation energy, the energy barrier of the reaction leading to products. This occurs because enzymes bind the high-energy intermediate of the reaction (the transition state) more strongly than the substrate. We studied details of this process by determining the substrate binding energy (ΔG_s, calculated from K_m values) and the activation energy (ΔG_T, determined from k_{cat}/K_m values) for the trypsin-catalyzed hydrolysis of oligopeptides. Plots of ΔG_T versus ΔG_s for oligopeptides with 15 amino acid replacements at each of the positions P_1', P_1, and P_2 were straight lines, as predicted by a derived equation that relates ΔG_T and ΔG_s. The data led to the conclusion that the trypsin active site has subsites that bind moieties of substrate and of transition state in characteristic ratios, whichever substrate is used. This was unexpected and means that each subsite characteristically favors substrate binding or catalysis. © 2002 Elsevier Science

Key Words: **activation energy; binding energy; catalysis; *Periplaneta americana;* subsite role; substrate specificity; transition state; trypsin.**

Enzymes are able to enhance reaction rates by factors of 10^9–10^{17} because they lower the energy of the most unstable species on the reaction pathway, the transition state. As the transition state energy level is reduced, the probability of its formation from the substrate is increased. This leads to an increase in the transition state concentration, resulting in the speed up of the chemical reaction. The energy lowering is mainly a consequence of enzymes binding to the transition state more strongly than to substrates (1). Hence, within the enzyme active site, the different amino acid residues may present a continuum of functions ranging from exclusive substrate binding to exclusive transition state binding (thus favoring catalysis) passing through residues with both functions (2, 3). In spite of these hypothesis, it is not known for any enzyme, the relative role of each subsite in catalysis and substrate binding and also, if such role is influenced by different substrates. Trypsin is a suitable model to address this problem. Trypsin cleaves polypeptide chains at the carboxyl side of basic L-amino acids such as arginine or lysine (4). Thus, using polypeptides containing arginine it is possible to define enzyme subsites that accommodate the amino acid residues at different positions in the substrate (5) (Fig. 1a). Substrate and transition state binding energies may be calculated for each trypsin subsite, by determining kinetic parameters for the trypsin hydrolysis of series of arginine-containing oligopeptides presenting changes in a single amino acid residue.

In this paper we show that trypsin active sites have subsites that are able to bind different moieties of substrates and transition states in ratios that are constant and characteristic of each subsite. The approach used is a useful tool to characterize the role of different enzyme subsites in catalysis and substrate binding and it provides a basis for the development of better substrates or inhibitors for endopeptidases and other enzymes acting on polymers.

MATERIALS AND METHODS

Trypsin purification. The experimental model was the *Periplaneta americana* (American cockroach) digestive trypsin purified after chromatographic steps consisting of an anion exchange in High Q column (Econo system, Bio-Rad, U.S.A.) followed by a second anion exchange in Mono Q column (FPLC system, Pharmacia Biotech, Sweden). The buffers of 20 mM piperazine adjusted to pH 5.0 or 10.0 were used in the High Q and Mono Q chromatographic steps, respectively. The trypsin was always eluted using a linear NaCl gradient ranging from 0 to 1.0 M. The trypsin (29 kDa) purity was ascertained by silver-stained SDS–PAGE (6).

Substrates. The substrates used were peptides having quenching (2,4-dinitrophenyl-ethylene diamine; EDDnp) and as a fluorescent (*ortho*-aminobenzoyl; Abz) groups at the C- and N-terminal ends, respectively, so that after hydrolysis the peptides become fluorescent (Fig. 1a). A total of 45 different substrates were synthesized (7) with

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TABLE 1

Effect of the Amino Acid Residue in Position P_3 on the Kinetic and Thermodynamic Parameters of the Substrate Hydrolysis by the Digestive Trypsin from *P. americana*

Position P_3	K_m (μM)	k_{cat} ($s^{-1} \cdot 10^{-4}$)	ΔG_s (kJ/mol)	$\Delta G_T^{\ddagger}$ (kJ/mol)
A	1.8 ± 0.2	3.3 ± 0.1	−33.3 ± 0.2	14.6 ± 0.3
I	1.7 ± 0.1	2.00 ± 0.03	−33.4 ± 0.1	15.8 ± 0.1
F	2.9 ± 0.4	3.0 ± 0.1	−32.1 ± 0.3	16.1 ± 0.3
E	3.7 ± 0.4	1.33 ± 0.06	−31.5 ± 0.2	18.8 ± 0.3
V	1.7 ± 0.1	2.17 ± 0.03	−33.4 ± 0.1	15.6 ± 0.1
S	2.4 ± 0.2	3.17 ± 0.06	−32.5 ± 0.2	15.5 ± 0.2
Q	3.1 ± 0.3	5.0 ± 0.1	−31.9 ± 0.2	15.0 ± 0.2
K	1.2 ± 0.1	4.1 ± 0.1	−34.3 ± 0.2	13.1 ± 0.2
D	7.3 ± 0.5	0.38 ± 0.01	−29.7 ± 0.1	23.6 ± 0.2
Y	2.4 ± 0.1	3.17 ± 0.05	−32.5 ± 0.1	15.5 ± 0.1
M	1.8 ± 0.1	4.1 ± 0.1	−33.3 ± 0.1	14.1 ± 0.1
L	2.5 ± 0.2	3.3 ± 0.1	−32.4 ± 0.1	15.5 ± 0.2
N	2.4 ± 0.2	1.63 ± 0.05	−32.5 ± 0.2	17.7 ± 0.2
H	2.2 ± 0.2	2.67 ± 0.08	−32.8 ± 0.3	15.7 ± 0.2
T	3.4 ± 0.2	1.65 ± 0.05	−31.7 ± 0.1	18.0 ± 0.1

Note. The substrate used in these determinations was *Abz*-A**X**GRGAGQ-*EDDnp* (Fig. 1a). **X** highlights the variable position (P_3).

TABLE 2

Effect of the Amino Acid Residue in Position P_2 on the Kinetic and Thermodynamic Parameters of the Substrate Hydrolysis by the Digestive Trypsin from *P. americana*

Position P_2	K_m (μM)	k_{cat} ($s^{-1} \cdot 10^{-6}$)	ΔG_s (kJ/mol)	$\Delta G_T^{\ddagger}$ (kJ/mol)
A	8 ± 2	2.0 ± 0.2	−29.5 ± 0.7	8.1 ± 0.6
I	40 ± 9	7 ± 1	−25.5 ± 0.6	8.8 ± 0.6
F	1.8 ± 0.3	1.08 ± 0.05	−33.3 ± 0.4	5.9 ± 0.4
E	9.1 ± 0.9	0.33 ± 0.01	−29.2 ± 0.2	12.9 ± 0.2
V	2.5 ± 0.6	1.8 ± 0.1	−32.4 ± 0.6	5.4 ± 0.5
S	5.1 ± 0.9	0.91 ± 0.03	−30.7 ± 0.4	8.9 ± 0.4
Q	11.0 ± 0.1	2.0 ± 0.1	−28.7 ± 0.1	8.9 ± 0.2
K	1.8 ± 0.6	0.117 ± 0.008	−33 ± 1	11.5 ± 0.7
D	3.8 ± 0.4	1.18 ± 0.01	−31.4 ± 0.2	7.5 ± 0.2
Y	1.8 ± 0.1	2.3 ± 0.2	−33.3 ± 0.1	3.9 ± 0.2
M	2.4 ± 0.4	1.8 ± 0.1	−32.5 ± 0.4	5.3 ± 0.4
L	1.7 ± 0.2	0.13 ± 0.01	−33.4 ± 0.3	11.0 ± 0.2
N	1.30 ± 0.09	0.40 ± 0.01	−34.1 ± 0.1	7.6 ± 0.1
H	0.50 ± 0.08	0.63 ± 0.05	−36.5 ± 0.4	4.0 ± 0.4
T	10 ± 1	0.41 ± 0.03	−29.0 ± 0.3	12.6 ± 0.3

Note. The substrate used in these determinations was *Abz*-AG**X**RGAGQ-*EDDnp* (Fig. 1a). **X** highlights the variable position (P_2).

15 amino acid replacements in each of the positions P_1', P_2, and P_3 (Fig. 1a).

Enzyme kinetic and thermodynamic parameters. The kinetic parameters $1/K_m$ (binding constant) and k_{cat}/K_m (apparent second-order rate constant) were calculated from the hydrolysis initial rate of 15 different concentrations of each substrate. For the P_2 and P_3 subsites characterization, the substrate concentration ranged from 1 to 20 μM and for the P_1' from 1 to 100 μM. All the enzymatic essays were performed at 30°C in 100 mM Tris–HCl pH 8.5 and the substrate hydrolysis was detected by Abz fluorescence (excitation 320 nm and emission 420 nm) (8).

The kinetic parameters were determined fitting the data into the Michaelis–Menten equation using the Enzfitter software (R. J. Leatherbarrow, Elsevier–Biosoft). These parameters were used to calculate the free energy of binding (ΔG_s) and the total free energy of activation ($\Delta G_T^{\ddagger}$) as follows:

$$\Delta G_s = -RT\ln 1/K_m$$

$$\Delta G_T^{\ddagger} = -RT\ln(k_{cat}/K_m) + RT\ln(K_B T/h),$$

where $T = 303$ K, R is the gas constant, K_B is the Boltzman constant, and h is Planck's constant.

RESULTS AND DISCUSSION

The enzyme kinetic data (K_m and k_{cat}) obtained from the hydrolysis of peptides series by the cockroach digestive trypsin were used to calculate the free energy of binding (ΔG_s) and activation energy ($\Delta G_T^{\ddagger}$) corresponding to substrate (Tables 1, 2, and 3). Within a series of substrates presenting a variable position occupied by different amino acids, the difference between those thermodynamic parameters is consequence of the different noncovalent interactions between the same enzyme subsite and the different amino acid occupying the substrate variable position. Thus, as a subsite may interact preferentially with substrate or transition state, the exchange of the amino acid interacting with that particular subsite may preferentially affect the substrate binding (ΔG_s) or activation energy ($\Delta G^{\ddagger}$).

TABLE 3

Effect of the Amino Acid Residue in Position P_1' on the Kinetic and Thermodynamic Parameters of the Substrate Hydrolysis by the Digestive Trypsin from *P. americana*

Position P_1'	K_m (μM)	k_{cat} ($s^{-1} \cdot 10^{-5}$)	ΔG_s (kJ/mol)	$\Delta G_T^{\ddagger}$ (kJ/mol)
A	3.7 ± 0.6	2.4 ± 0.1	−31.5 ± 0.4	11.5 ± 0.4
I	5.9 ± 0.7	1.33 ± 0.05	−30.3 ± 0.3	14.1 ± 0.3
F	4.1 ± 0.6	2.17 ± 0.08	−31.2 ± 0.3	12.0 ± 0.4
E	18 ± 1	1.33 ± 0.05	−27.5 ± 0.2	17.0 ± 0.2
V	4.0 ± 0.7	1.33 ± 0.06	−31.3 ± 0.4	13.2 ± 0.5
G	3.5 ± 0.7	2.0 ± 0.1	−27.5 ± 0.2	11.8 ± 0.5
Q	3.9 ± 0.6	2.3 ± 0.1	−31.3 ± 0.4	11.7 ± 0.4
S	8.5 ± 0.7	4.0 ± 0.1	−29.4 ± 0.2	12.3 ± 0.2
K	2.0 ± 0.1	2.3 ± 0.1	−33.0 ± 0.1	10.0 ± 0.2
D	8 ± 1	1.23 ± 0.05	−29.5 ± 0.3	15.1 ± 0.3
Y	4.0 ± 0.4	1.67 ± 0.05	−31.3 ± 0.2	12.6 ± 0.2
M	2.5 ± 0.4	2.00 ± 0.06	−32.4 ± 0.4	11.0 ± 0.4
L	3.6 ± 0.4	1.38 ± 0.05	−31.5 ± 0.2	12.8 ± 0.3
N	6.2 ± 0.4	3.00 ± 0.06	−30.2 ± 0.1	12.2 ± 0.1
H	9.8 ± 0.6	1.83 ± 0.05	−29.0 ± 0.1	14.6 ± 0.1
T	4.3 ± 0.2	2.00 ± 0.03	−31.1 ± 0.1	12.3 ± 0.1

Note. The substrate used in these determinations was *Abz*-AGGR**X**AGQ-*EDDnp* (Fig. 1a). **X** highlights the variable position (P_1').

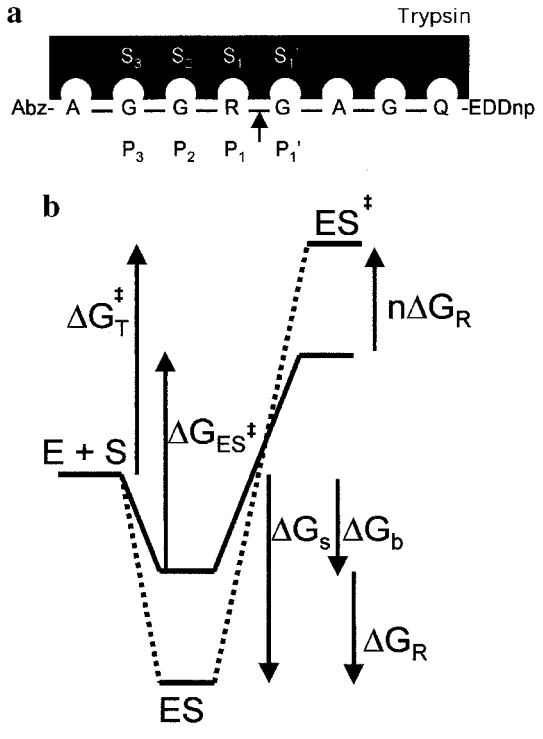

FIG. 1. Relationship between activation and binding energies of trypsin substrates. (a) Substrates like Abz-AGGRGAGQ-EDDnp were synthesized with 15 amino acids replacements at each of the positions P_1', P_2, and P_3. The arrow indicates the bond cleaved by trypsin. Abz, *ortho*-aminobenzoyl; EDDnp, *N*-(2,4-dinitrophenyl)-ethylene diamine. (b) Gibbs free energy changes associated with oligopeptide hydrolysis. The solid line corresponds to energy changes associated to the chain shared with all oligopeptides (the dashed line includes also amino acid radicals) in the enzyme–substrate complex in the ground (ES) or in the transition state ($ES^{\ddagger}$). $\Delta G_T^{\ddagger}$, total activation energy; $\Delta G_{ES}^{\ddagger}$, activation energy of the chemical steps of bond making and breaking of the shared chain of oligopeptides differing in a single amino acid at a single position; ΔG_R, contribution of the radical part of the amino acid in consideration (R) to binding and activation energy; ΔG_S, binding energy of a particular oligopeptide including the binding energy of the shared chain (ΔG_b) and of R (ΔG_R); *n*, ratio of R binding energies in $ES^{\ddagger}$ and ES.

To quantify the subsite preference, it was deduced a mathematical relationship (Eq. [3], see below) between total activation energy ($\Delta G_T^{\ddagger}$) and substrate binding energy (ΔG_s) for the series of trypsin substrates (Tables 1, 2, and 3). The equation was derived from the Gibbs free energy profile associated with the oligopeptides hydrolysis by trypsin (Fig. 1b). The oligopeptides belonging to the same substrate series share most of their structure, except the side chain (R) of the amino acid in the variable position. Thus, the binding energy of a particular oligopeptide includes the binding energy of the shared part of the structure (ΔG_b) and the binding energy of the variable side chain R (ΔG_R). Thus, from the Fig. 1b,

$$\Delta G_S = \Delta G_b + \Delta G_R. \qquad [1]$$

Moreover, as the side chain (R) may interact differentially with the trypsin subsite in the ES and $ES^{\ddagger}$, it was introduced the factor "*n*" that represents the ratio of R binding energies between $ES^{\ddagger}$ and ES. Thus, the total activation energy may be written

$$\Delta G_T^{\ddagger} = \Delta G_{ES}^{\ddagger} + n\Delta G_R - \Delta G_R + \Delta G_S. \qquad [2]$$

Combining Eq. [1] and Eq. [2] results in

$$\Delta G_T^{\ddagger} = \Delta G_{ES}^{\ddagger} + n(\Delta G_s - \Delta G_b) - (\Delta G_s - \Delta G_b) + \Delta G_s$$

$$\Delta G_T^{\ddagger} = \Delta G_{ES}^{\ddagger} + n\Delta G_s - n\Delta G_b - \Delta G_s + \Delta G_b + \Delta G_s$$

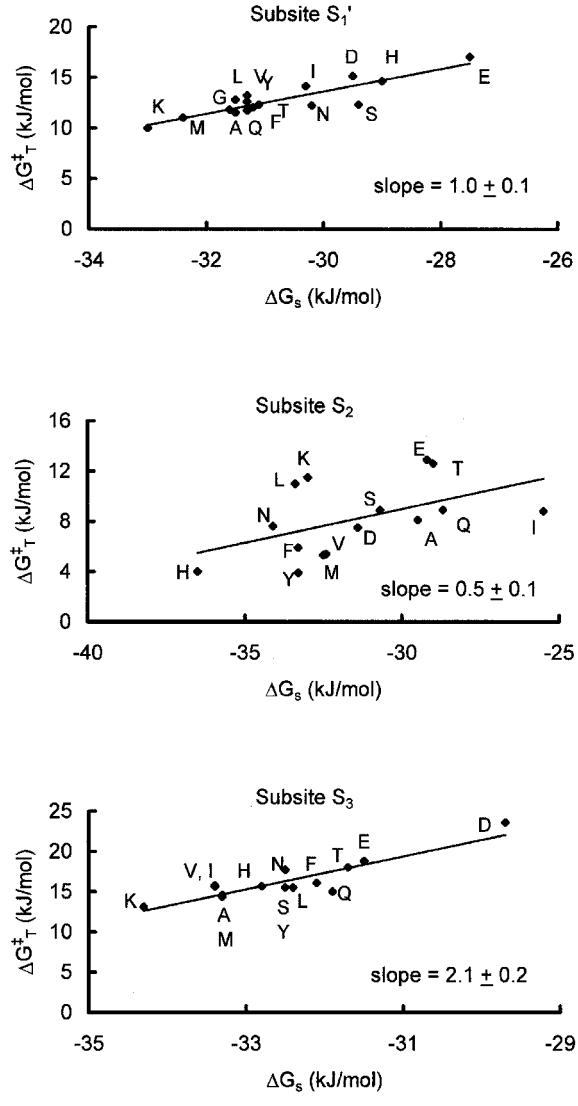

FIG. 2. Total activation energies ($\Delta G^{\ddagger}{}_T$) versus substrate binding energies (ΔG_S) for *P. americana* trypsin subsites. S_1', S_2, and S_3 are trypsin subsites that correspond to positions indicated in the oligopeptide (Fig. 1a). Each point in the curve represents a substrate in which one amino acid (identified by a letter) has been changed.

$$\Delta G_T^{\ddagger} = \Delta G_{ES}^{\ddagger} + \Delta G_b(1 - n) + n\Delta G_S. \quad [3]$$

As it can be seen, the Eq. [3] describes a straight line with $\Delta G_{ES}^{\ddagger} + \Delta G_b(1 - n)$ as intercept and n as slope, respectively. The slope "*n*" is the ratio of transition state and substrate binding energy of the moiety R (Fig. 1b), thus indicating what the corresponding subsite favors: catalysis or substrate binding. A subsite involved only in substrate binding should present $n = 0$, whereas a subsite involved only in transition state binding would be expected to have $n = +\alpha$. If *n* varies between 0 and $+\alpha$, this indicates a subsite involved in both substrate and transition state binding. In this case, the *n* value would indicate the major role of the subsite:substrate binding ($n < 1$) or transition state binding ($n > 1$).

As we can see (Fig. 2), the plots of $\Delta G_T^{\ddagger}$ versus ΔG_s are straight lines, as predicted by Eq. [3]. Subsite S_3 of *P. americana* trypsin has $n = 2.1$, indicating that it binds the transition state with double the energy required to bind the substrate. Subsite S_1' has $n = 1.0$, showing that it binds both substrate and transition state with similar energy. In this case, the subsite contributes equally to substrate binding and catalysis. Subsite S_2 have n = 0.6, revealing that it binds the substrate more tightly than the transition state.

This means that in *P. americana* trypsin, the subsites S_3 contributes more to catalysis than to substrate binding, whereas the contrary is true for subsite S_2. Furthermore, the presence of linear relationships with slope higher than 0 and lower than $+\alpha$ suggests that the binding interactions in the ES and $ES^{\ddagger}$ complexes are not isolated from each other, as would be expected if n equal to was 0 (binding only substrate) or $+\alpha$ (binding only the transition state).

Finally, results presented here led us to conclude that the preference for binding moieties of the substrate or transition state is a substrate-independent property of each trypsin subsite. In other words, each trypsin subsite favors catalysis or substrate binding in a characteristic way.

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