The positive electrostatic environment of the active site of prolyl oligopeptidase was investigated by using substrates with glutamic acid at positions P2, P3, P4, and P5, respectively. The different substrates gave various pH rate profiles. The $pK_a$ values extracted from the curves are apparent parameters, presumably affected by the nearby charged residues, and do not reflect the ionization of a simple catalytic histidine as found in the classic serine peptidases like chymotrypsin and subtilisin. The temperature dependence of $k_{cat}/K_m$ did not produce linear Arrhenius plots, indicating different changes in the individual rate constants with the increase in temperature. This rendered it possible to calculate these constants, $i.e.$ the formation ($k_1$) and decomposition ($k_2$) of the enzyme-substrate complex and the acylation constant ($k_3$), as well as the corresponding activation energies. The results have revealed the relationship between the complex Michaelis parameters and the individual rate constants. Structure determination of the enzyme-substrate complexes has shown that the different substrates display a uniform binding mode. None of the glutamic acids interacts with a charged group. We conclude that the specific rate constant is controlled by $k_1$ rather than $k_2$ and that the changes from the substrate and the enzyme can markedly affect the formation but not the structure of the enzyme-substrate complexes.

Prolyl oligopeptidase is a member of a relatively new group of serine peptidases unrelated to the well known trypsin and subtilisin families (1–4). The new family includes enzymes of different specificities like the prolyl oligopeptidase itself, dipeptidyl-peptidase IV, acylaminoacyl-peptidase, and oligopeptidase B (5). These enzymes selectively cleave substrates that are no longer than $\sim$30 amino acid residues in total. Prolyl oligopeptidase (EC 3.4.21.26) is implicated in the metabolism of peptide hormones and neuropeptides (6–8). Because specific inhibitors relieve scopolamine-induced amnesia (9–12), the enzyme is of pharmaceutical interest. The activity of prolyl oligopeptidase has also been associated with depression (13, 14) and blood pressure regulation (15).

The crystal structure determination of prolyl oligopeptidase has revealed that the carboxyl-terminal peptidase domain of the enzyme displays an $\alpha/\beta$ hydrolase fold and that its catalytic triad (Ser-554, His-680, and Asp-641) is covered by the central tunnel of an unusual $\beta$-propeller (16). Recent engineering of the enzyme provided evidence for a novel strategy of regulation in which oscillating propeller blades act as a gating filter during catalysis, letting small peptide substrates into the active site while excluding large proteins, thereby preventing accidental proteolysis in the cytosol (17).

The active site region of prolyl oligopeptidase exhibits several charged residues such as Arg-643, Asp-642, Arg-252, Asp-149, and Arg-128. The complex electrostatic environment created by these residues may considerably influence the binding and thus the specificity of substrates. We have determined previously that, principally, five subsites of the enzyme (S3–S2') interact with a polypeptide substrate (18). In this work we have examined the effects of charged residues at different subsites. Because of the mainly positive environment around the active site, we substituted glutamic acid for the residues of the internally quenched substrate Abz-Gly-Phe-Ser-Pro-Phe(NO$_2$)-Ala, as the leader peptide, where Abz and Phe(NO$_2$) were the fluorescent donor and the quencher, respectively.

### EXPERIMENTAL PROCEDURES

**Enzyme Preparations**—Prolyl oligopeptidase from porcine brain and its variants S554A and R252S were expressed in *Escherichia coli* JM105 cells and purified as described previously (19, 20). The enzyme concentrations were determined at 280 nm (3).

**Kinetics**—The reaction of prolyl oligopeptidase with Z-Gly-Pro-Nap (Bachem Ltd., Bubendorf, Switzerland) was measured fluorometrically using a Cary Eclipse fluorescence spectrophotometer equipped with a Peltier four-position multicell holder accessory and a temperature controller. The excitation and emission wavelengths were 340 and 410 nm, respectively. Cells with excitation and emission path lengths of 1 and 0.4 cm, respectively, were used. The substrates with internally quenched fluorescence, Abz-Gly-Phe-Ser-Pro-Phe(NO$_2$)$_2$-Ala and its derivatives, were prepared with solid phase synthesis, and their hydrolyses were followed as in the case of Z-Gly-Pro-Nap, except that the excitation and emission wavelengths were 357 and 420 nm, respectively.

The pseudo-first order rate constants were measured at substrate concentrations lower than 0.1 $K_m$ and calculated by non-linear regression data analysis using the GraFit software (21).
constants \(k_{cat}/K_m\) were obtained by dividing the pseudo-first order rate constant by the total enzyme concentration in the reaction mixture.

The Michaelis-Menten parameters \(k_{cat}\) and \(K_m\) were determined with initial rate measurements using substrate concentrations in the \(K_m\) value range of 0.2–5. The kinetic parameters were calculated with nonlinear regression analysis. With substrates exhibiting very low \(K_m\) values, initial rates below the \(K_m\) could not be measured; therefore, the parameters were calculated from a single progress curve using the integrated Michaelis-Menten equation (22, 23).

Theoretical curves for the bell-shaped pH rate profiles were calculated by nonlinear regression analysis using Equation 1 and the GraFit software (21). In Equation 1, shown here,

\[
h_{cat}/K_m = h_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(1)})} + 10^{pH - pK_{cat(2)})})
\]

\(h_{cat}/K_m(\text{limit})\) stands for the pH-independent maximum rate constant, and \(K_{cat}\) and \(K_m\) are the dissociation constants of the catalytically competent base and acid, respectively. When an additional ionizing group modifies the bell-shaped character of the pH dependence curve, the data were fitted to Equation 2 (doubly bell-shaped curve), shown here,

\[
h_{cat}/K_m = h_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(1)})} + 10^{pH - pK_{cat(2)})}) + h_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(2)})} + 10^{pH - pK_{cat(3)})})
\]

Equation 2 also applies for two active forms of the enzyme, and \(K_1, K_2, K_3\), and \(K_4\) are the apparent dissociation constants of the enzymatic groups whose state of ionization controls the rate constants. The points for the rate constants for the substrate with Arg at position P2 were fitted to Equation 3, depicted here,

\[
h_{cat}/K_m = h_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(1)})} + 10^{pH - pK_{cat(2)})}) + h_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(2)})} + 10^{pH - pK_{cat(3)})})
\]

which is composed of a bell-shaped and a sigmoid term.

**Extraction of Individual Rate Constants from Nonlinear Temperature Dependence**—Deviation from the linearity of an Arrhenius plot may be indicative of changes in rate-limiting steps, which allows for resolution of the individual rate constants that compose \(k_{cat}/K_m\) as defined by Equations 4 and 5 (24, 25), shown here,

\[
\frac{k_1}{k_2} = \frac{E + S \rightarrow ES \rightarrow EA}{k_{-1}}
\]

\(k_{cat}/K_m = k_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(1)})} + 10^{pH - pK_{cat(2)})}) + k_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(2)})} + 10^{pH - pK_{cat(3)})})
\]

where \(k_1\) and \(k_{-1}\) are the rate constants for binding and dissociation of the substrate, \(k_2\) is the first-order acylation rate constant, \(EA\) is the acyl enzyme, and \(\alpha = k_{cat}/K_m\), measures the stickiness of the substrate (26), which indicates that the substrate dissociates more slowly from its complex formed with the enzyme than it reacts to yield product (i.e. stickiness is high if \(k_{cat}/K_m\) approaches \(k_{-1}\) whenever \(\alpha > 1\)). The temperature dependence of the rate constants can be obtained from Equation 6 (24), presented here,

\[
h_{cat}/K_m = h_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(1)})} + 10^{pH - pK_{cat(2)})}) + h_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(2)})} + 10^{pH - pK_{cat(3)})})
\]

Equation 6 also applies for two active forms of the enzyme, and \(K_1, K_2, K_3\), and \(K_4\) are the apparent dissociation constants of the enzymatic groups whose state of ionization controls the rate constants. The points for the rate constants for the substrate with Arg at position P2 were fitted to Equation 3, depicted here,

\[
h_{cat}/K_m = h_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(1)})} + 10^{pH - pK_{cat(2)})}) + h_{cat}/K_m(\text{limit}) \times (1/(1 + 10^{pH - pK_{cat(2)})} + 10^{pH - pK_{cat(3)})})
\]

which is composed of a bell-shaped and a sigmoid term.
zyme (16). Crystals belong to the orthorhombic space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}. X-ray diffraction data were collected using synchrotron radiation. Data were processed using the HKL suite of programs (27). Refinement of the structures was carried out by alternate cycles of REFMAC (28) and manual refitting using O (29) based on the 1.4-Å resolution model of wild type enzyme (16) (Protein Data Bank code 1QFM). Water molecules were added to the atomic model automatically using ARP (30) at the positions of large positive peaks in the difference electron density, but only at places where the resulting water molecule fell into an appropriate hydrogen-bonding environment. Restrained isotropic temperature factor refinements were carried out for each individual atom. Data collection and refinement statistics are given in Table I.

**Table I**

| Parameter | P2 Arg | P2 Glu | P3 Glu | P4 Glu | P5 Glu |
|-----------|--------|--------|--------|--------|--------|
| $k_{\text{cat}}$ (s$^{-1}$) | 3.20 ± 0.09 | 2.40 ± 0.03 | 1.82 ± 0.03 | 1.70 ± 0.07 | 7.22 ± 0.21 |
| $K_m$ (µM) | 0.40 ± 0.07 | 1.54 ± 0.04 | 6.3 ± 0.2 | 1.32 ± 0.12 | 3.7 ± 0.3 |
| $k_{\text{cat}}/K_m$ (µM$^{-1}$ s$^{-1}$) | 8 ± 1.4 | 1.55 ± 0.08 | 0.26 ± 0.01 | 1.56 ± 0.15 | 1.95 ± 0.17 |
| $k_{\text{cat}}/K_m$(lim)$_1$ (µM$^{-1}$ s$^{-1}$) | 3.3 ± 1.1 | 1.2 ± 0.2 | 1.3 ± 0.3 |
| $k_{\text{cat}}/K_m$(lim)$_2$ (µM$^{-1}$ s$^{-1}$) | 10.4 ± 0.4 | 3.4 ± 0.2 | 2.9 ± 0.2 | 2.87 ± 0.03 |
| pH$_K$ | 5.8 ± 0.5 | 5.1 ± 0.5 | 5.37 ± 0.07 | 4.7 ± 1.0 | 4.5 ± 0.1 |
| pH$_2$ | 7.6 ± 0.2 | 7.1 ± 0.2 | 7.1 ± 0.3 |
| pH$_3$ | >9 | 8.91 ± 0.08 | 8.73 ± 0.05 | 8.94 ± 0.09 | 8.68 ± 0.02 |
| pH optimum | >8.5 | 7.9 | 7 | 7.9 | 6.6 |
| $\Delta H^\circ$ (kJ/mol) for $k_1$ | 115.6 | 72.4 | 102.2 | 80.7 | 106 |
| $\Delta S^\circ$ (J/mol K) for $k_1$ | 274 | 120 | 202 | 148 | 238 |
| $\Delta G^\circ$ (kJ/mol) for $k_1$ | 33.8 | 36.4 | 42 | 36.4 | 35.1 |
| $\Delta H^\circ$ (kJ/mol) for $k_{\text{cat}}$ | 48.7 | 85.7 | 85.7 | 71.3 |
| $\Delta S^\circ$ (J/mol K) for $k_{\text{cat}}$ | −74 | 45.1 | 45.1 | 9.7 |
| $\Delta G^\circ$ (kJ/mol) for $k_{\text{cat}}$ | 70.8 | 72.3 | 72.3 | 68.5 |

**Fig. 1.** The pH dependences of $k_{\text{cat}}/K_m$. A, P2 Arg (○, left ordinate); the points were fitted to Equation 3, and the broken line is a simple sigmoid curve. P2 Glu (○, right ordinate); the points were fitted to Equation 2, and the broken line represents a bell-shaped curve. B, P3 Glu (+, right ordinate), P4 Glu (∆, left ordinate), and P5 Glu (○, left ordinate).
The Effects of Arg252 on Specificity Rate Constants for Prolyl Oligopeptidase

The reactions were measured in the absence or in the presence of 0.5 M NaCl at substrate concentrations of 0.2-0.3 μM. The numbers in brackets stand for the pH values close to the pH optimum where the $k_{cat}/K_m$ values were determined. The amino acid residues of the leader peptide, Abz-Gly-Phe-Ser-Pro-Phe(NO$_2$)-Arg-Ala, are substituted at the P3 and P4 positions, respectively, by a glutamic acid residue. P5 Glu = Abz-Glu-Gly-Pro-Phe-Gly-Phe(NO$_2$)-Ala.

**Table III**

| Enzyme            | P3 Glu | P4 Glu | P5 Glu | Z-Gly-Pro-Nap |
|-------------------|--------|--------|--------|---------------|
|                   | $μM^{-1}s^{-1}$ | $μM^{-1}s^{-1}$ | $μM^{-1}s^{-1}$ | $μM^{-1}s^{-1}$ |
| Wild type         | 0.332 (7) | 1.81 (7) | 3.09 (6.5) | 4.84 (8) |
| R252S variant     | 0.129 (6.5) | 1.50 (7.5) | 1.62 (7) | 3.77 (7.5) |
| Wild type (NaCl)  | 0.575 (7.6) | 5.50 (7.6) | | |
| R252S variant (NaCl) | 0.125 (7) | 1.14 (7.8) | | |

**Fig. 2.** The pH rate profiles for the reactions of prolyl oligopeptidase (Δ) and its R252S variant (○) with the substrate containing P3 Glu.

**Fig. 3.** Arrhenius plots for $k_{cat}/K_m$. Shown are Arg at the P2 position (A) and Glu at the P3 position (B) of the leader peptide Abz-Gly-Phe-Ser-Pro-Phe(NO$_2$)-Arg-Ala. The broken lines were calculated with the $k_1$ and $E_1$ values shown in Table IV.

**RESULTS AND DISCUSSION**

The Effects of Glu at Various P Sites—We have shown previously that succinyl-Gly-Pro-4-nitroanilide is much worse as a substrate than Z-Gly-Pro-Nap (20). In light of the basic environment of the active site, a rationale for the large difference may be the effect of the negative charge of the succinyl group. Therefore, we examined the effects on the kinetic and thermodynamic parameters of the glutamic acid residue located at various sites of the leader peptide, Abz-Gly-Phe-Ser-Pro-Phe(NO$_2$)-Arg-Ala, which corresponds to the P1-P4 amino acids of bradykinin, a possible natural substrate. Our previous x-ray crystallographic studies on the binding of an octapeptide indicated that the P3-P2′ residues were clearly associated with the enzyme, whereas the P4 residue might bind weakly (18). Yet, we have also extended the position of Glu toward subsites 4 and 5, because it is known from the three-dimensional structure of prolyl oligopeptidase (20) that Arg-252 and Arg-128 are not far from the binding site so that they may form appropriate subsites for the binding of Glu in those positions. We have also analyzed the effects of the opposite charge by using Arg at the P2 position.

Fig. 1 illustrates the pH dependences of $k_{cat}/K_m$ for the substrates containing P2 Arg and P2, P3, P4, and P5 Glu residues. The P2 Arg has a high pH optimum. The pH dependence does not follow a simple ionization curve, as indicated by the points deviated at low pH. The reaction of the peptide with a Glu residue at positions P3, P4, or P4, in particular at P4, conforms to a double bell curve, whereas at positions P3 and P5 simple bell-shaped curves are displayed. The acidic limb of the pH dependence curve for substrates with Glu is shifted toward the lower pH region. The further the Glu is from the scissile bond, the greater the shift. The parameters of the pH dependence curves are shown in Table II, which also shows the kinetic parameters $k_{cat}$ and $K_m$. The $K_m$ is highest for the peptide with P3 Glu and lowest with P2 Arg. This suggests that poor binding makes the peptide with P3 Glu the worst substrate of this series, whereas the best substrate that possesses a P2 Arg exhibits the strongest binding. The differences in the $k_{cat}$ values are less important. It may be noted that the carboxyl-terminal carboxyl group of the substrate does not affect the pH rate profiles because its ionization is outside of the pH range studied. Moreover, we did not observe any difference between the leader peptide and its amide derivative (not shown).

Kinetic Properties of the R252S Variant—Possible effects of Arg252 on the kinetic parameters of the different substrates were examined by using the R252S variant, which eliminates a positive charge near the binding site. As seen from Table III it is the P3 Glu that is most affected, in particular in the presence of 0.5 M NaCl. Interestingly, the rate constant for the P4 Glu substrate is not affected, and the rate constant for the neutral Z-Gly-Pro-Nap is only moderately decreased. Fig. 2 shows the pH dependence of $k_{cat}/K_m$ for the P3 Glu substrate. It is seen that not just the value of the specificity rate constant but also its pH dependence has changed considerably. The alterations...
A deeper insight into the kinetic specificity of prolyl oligopeptidase, and Glu at the P5 (at the P3 (24, 25). However, the moderate decline with P2 Glu did not permit us to extract precise rate constants that compose $k_{\text{cat}}$ and $K_{\text{m}}$ values from the curve. Unfortunately, above 40 °C the enzyme tended to denature, which prevented the accurate determination of the rate constants by using higher temperatures.

It should be noted that conformational changes might also result in nonlinear Arrhenius plots (31, 32). For example, sharp changes in slope were found at about 14 °C for d-amino acid oxidase; at the same temperature, sedimentation velocity and UV spectroscopy indicated a change in conformational state of the enzyme (31). In the present case, however, the data points fit well to the theoretical curve, which is not expected when conformational changes predominate. In addition, the activation energies seem to be independent of temperature as required in the short temperature range employed here, whereas reactions with different enzyme conformations could proceed with different activation energies. It is likely, however, that conformational changes are associated with the change in the rate constants. A further problem may arise from the cis-trans isomerization of proline-containing substrates (3, 33), which may also change with the increase of temperature. However, our experimental conditions were not rate-determining for cis-trans isomerization (3).

Fig. 4. Arrhenius plots for $k_{\text{cat}}$. Shown are Arg at the P2 ($\times$), Glu at the P3 (○), and Glu at the P5 (□) positions.

TABLE IV

| Parameter | 15 °C | 20 °C | 25 °C | 30 °C | 35 °C |
|-----------|-------|-------|-------|-------|-------|
| $k_1$ ($\mu$m$^{-1}$ s$^{-1}$) | 1.5 ± 0.1 | 3.5 ± 0.5 | 7.9 ± 1.5 | 17 ± 4 | 36 ± 9 |
| $k_2$ | 5 ± 2 | 2 ± 1 | 1.1 ± 0.4 | 0.6 ± 0.2 | 0.3 ± 0.1 |
| $E_1$ (kJ/mol) | 118 ± 9 | 120 ± 9 | 120 ± 9 | 118 ± 8 | 118 ± 8 |
| $E_{-1} - E_a$ (kJ/mol) | 104 ± 5 | 104 ± 5 | 104 ± 5 | 104 ± 5 | 104 ± 6 |

TABLE V

| Parameter | P2 Arg (pH 8.6) | P3 Glu (pH 7) | P5 Glu (pH 7) |
|-----------|----------------|---------------|---------------|
| $k_1$ ($\mu$m$^{-1}$ s$^{-1}$) | 7.9 ± 1.6 | 0.27 ± 0.10 | 2.9 ± 0.7 |
| $k_{-1}$ (s$^{-1}$) | 2.3 ± 0.1 | 0.49 ± 0.04 | 2.9 ± 0.2 |
| $E_1$ (kJ/mol) | 120 ± 9 | 105 ± 18 | 120 ± 12 |
| $E_{-1}$ (kJ/mol) | 163 ± 3 | 195 ± 7 | 190 ± 4 |
| $K_{m} = k_{-1}/k_1$ (µM) | 0.29 | 1.81 | 0.99 |

were less impressive with P4 Glu and P5 Glu. Nonetheless, it is clear from Table III and Fig. 2 that the electrostatic environment created by Arg-252 does influence the catalysis.

Temperature Dependence of Rate Constants—To gain a deeper insight into the kinetic specificity of prolyl oligopeptidase, we have also investigated the temperature dependence of the catalysis. For each substrate the Arrhenius plot deviated from a straight line as shown in Fig. 3 for the substrates containing P2 Arg (Fig. 3A) and P3 Glu (Fig. 3B). It should be emphasized that the decline in the curve with the increase in temperature was not due to denaturation. Precipitation of the enzyme at high temperature for a longer time than that required for the activity measurement did not affect the value of the rate constant. The deviations from linearity were different for the various substrates, i.e. smaller with P2 Glu and greater with P2 Arg and P5 Glu, with P3 Glu and P4 Glu being intermediates. The nonlinear Arrhenius plot rendered it possible to determine the individual rate constants that compose $k_{\text{cat}}$ and $K_{\text{m}}$ (24, 25). However, the moderate decline with P2 Glu did not permit us to extract precise $k_2/k_1$ values from the curve. Unfortunately, above 40 °C the enzyme tended to denature, which prevented the accurate determination of the rate constants by using higher temperatures.
The kinetic \((k_1, k_2/k_3)\) and activation \((E_1, E_1 - E_2)\) parameters calculated from the temperature dependence of \(k_{cat}/K_m\) are listed in Table IV, which shows two interesting phenomena. First, the \(k_1\) is close to the specificity rate constant, independent of its value, and this suggests that the rate-limiting step is the formation of the enzyme-substrate complex. This may be surprising, because usually the substrate binding is a very fast, diffusion-controlled process. In the present case, it appears that the nature of substrate controls the access to the active site. Secondly, the \(k_1\) values are not consistent with their activation energies \((E_1)\). For example, the higher \(k_1\) for P2 Arg is associated with a higher \(E_1\) compared with the \(E_1\) for the Glu-containing substrates having lower \(k_1\) values. Because the faster reaction displays too high an activation energy, the activation entropy should compensate for the unexpected effect.

The activation parameters \((\Delta H^o \text{ and } \Delta S^o)\) were calculated.
from Eyring plots (ln(k/T) versus 1/T), using the k values of Table IV. The plots gave perfectly straight lines (not shown), and the calculated parameters are shown in Table II. Indeed, the ΔS* is higher for the substrate having the P2 Arg compared with the ΔS* for the slower reactions of the Glu-containing substrates. Most importantly, the ΔS* values are positive for all substrates, which indicates that the transition states of the reactions are less ordered than the ground states, an atypical phenomenon in enzyme catalysis. The release on binding of the ordered water molecules that are associated with the substrates and the enzyme can account for the greatest portion of the highly positive values.

Table II also shows ΔH* and ΔS* for kcat, which is characteristic of the breakdown of the enzyme-substrate complex and does not principally involve the removal of water molecules. As expected, a negative ΔS* was obtained with the substrate containing P2 Arg. However, the rate constants for the negatively charged compounds display moderately or slightly positive values. Possibly, the enzyme-substrate complexes that include glutamic acids are more disordered, and their motions must be reduced. Possibly, the enzyme-substrate complexes that include charged compounds display moderately or slightly positive values. It may be noted that the remaining activity is not due to the active enzyme contaminating the inactive form. This is consistent with the highest Kcat value for this substrate (Table II). The weak binding of the substrate with P3 Glu is reasonable in terms of the hydrophobic S3 subsite (which has a preference for Phe) and large hydrophobic groups such as the benzyloxycarbonyl group, but it disfavors the succinyl group, although this charged group binds at the same place as the aromatic ring (20).

Like prolyl oligopeptidase, the classical serine proteases also lack interactions between enzyme groups and substrate side chains. Apart from the primary specificity determinant (P1 residue), interactions tend to be between the enzyme and the substrate main chain groups (35).

The uniform binding of the different substrates is apparently at variance with the significant differences in rate constants and their dissimilar pH dependences. This indicates that substrate specificity is not entirely controlled by the binding mode but is also dependent on the route to the active site, which is markedly influenced by the charged residues of the substrate and the enzyme.

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