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A Mutational Approach Shows Similar Mechanisms of Recognition for the Isolated and Integrated Versions of a Protein Epitope*

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Antibody mAb164 is directed against the native form of the $TrpB_2$ subunit of *Escherichia coli* tryptophan synthase. It recognizes a synthetic peptide, P11, constituted of residues 273–283 of TrpB, with high affinity. We introduced 16 single and 3 double mutations in each of the two contexts, $TrpB_2$ and P11, and used them as local probes to study the cross-reactivity of mAb164 toward these two antigens. The equilibrium constant, K_D, of dissociation from mAb164 was measured for each of the mutant derivatives of $TrpB_2$ and P11 by a competition enzyme-linked immunosorbent assay and compared with the wild type one. The variation of the free energy of interaction, ΔΔG, covered nearly 8 kcal/mol for the different mutations. The values of ΔΔG for the mutant derivatives of $TrpB_2$ and for those of P11 were close and the two sets of values were strongly correlated ($r = 0.96$). This correlation showed that mAb164 recognized the integrated and isolated versions of residues 273–283 with very similar mechanisms. A few significant differences between the recognitions of $TrpB_2$ and P11 by mAb164 suggested some adaptability of the interaction. The results were compatible with a recognition of residues 273–283 of TrpB in a loop conformation, close to their structure in the crystals of the complete tryptophan synthase, $TrpA_2TrpB_2$.

Cross-reactivity of an antibody toward a protein and a peptide is the ability for an antibody directed against a protein to recognize a derived peptide and, reciprocally, for an antibody directed against a peptide to recognize the protein from which it is derived (1). This phenomenon is not limited to antibodies, and it exists for other types of receptors (2). Its observation constitutes the empirical basis for the design or selection of peptides that mimic full-length proteins and for their use as synthetic vaccines, inhibitors, or, more generally, new pharmaceuticals (3–6).

A better understanding of the molecular mechanisms that underlie the cross-reactivity toward related proteins and peptides could provide rational bases for the design of useful peptides. Ideally, the three-dimensional structures of the free parental protein, of the free derived peptide, and of their respective complexes with the antibody would be necessary for the analysis of these mechanisms. However, such a set of structural data has not been obtained so far. The best analyses of cross-reactivity have been obtained by comparing the structures of complexes between antibodies and peptides with those of the free parental proteins from which the peptides were derived (7–13). We used a different approach to this problem, in which mutations were introduced into both contexts, the parental protein and the derived peptide, and used as local probes of the interaction with the antibody.

mAb164[1] is a mouse monoclonal antibody that is directed against one subunit (the homodimer $TrpB_2$) of the tryptophan synthase from *Escherichia coli* (Fig. 1). The equilibrium dissociation constant, K_D, of their complex is equal to 0.2 nM (14). mAb164 recognizes a synthetic peptide, which is constituted of residues 273–283 of TrpB and called P11, with high affinity (K_D = 7 nM) (15). The crystal structure of the complete tryptophan synthase (the heterotetramer $TrpA_2TrpB_2$) is known, but not the structure of the free form of $TrpB_2$, which was used as immunogen to raise mAb164. Residues 273–283 of TrpB form a hairpin in the structure of $TrpA_2TrpB_2$. Eight of them belong to the interface between TrpA and $TrpB_2$, so their conformations could be different in $TrpA_2TrpB_2$ and in the free $TrpB_2$ (16, 17). A conformational analysis of the isolated synthetic peptide P11 by proton NMR spectroscopy has shown that its molecules, in the majority, adopt an extended conformation but that some of them, in the minority, are structured in their C-terminal part and comprise at least two different conformers (18).

In a previous work, we analyzed the mechanism of recognition between the isolated peptide P11 and antibody mAb164 through a mutational approach. We have constructed a fusion protein, MalE-P11, between protein MalE from *E. coli* and P11 at the genetic level and checked that P11 has the same conformational and functional properties in the context of a synthetic undecapeptide and in the context of hybrid MalE-P11 (19). We have introduced about 30 single and double mutations individually in MalE-P11, measured the K_D values for the interaction between the MalE-P11 variants and mAb164 by a competition enzyme-linked immunosorbent assay, and compared the K_D values. We have thus shown that mAb164 recognizes P11 in a loop conformation, close to that of residues 273–283 of TrpB in the crystal structure of $TrpA_2TrpB_2$. A comparison of the NMR data on the conformation of the isolated peptide P11 with the kinetic and mutational data on its recognition by mAb164 has indicated that mAb164 selects a conformer of P11 that represents only a small minority of the molecules (20).

In the present work, we compared the mechanisms of recognition by mAb164 for the isolated and integrated versions of the epitope, *i.e.* for $TrpB_2$ and P11. To do so, we used 19 single and double mutations of residues 273–283 of TrpB as local probes. We had previously introduced these mutations in hybrid MalE-P11. We also introduced them in subunit $TrpB_2$ and

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[1] The abbreviations used are: mAb, monoclonal antibody; wt, wild type; mut, mutant.

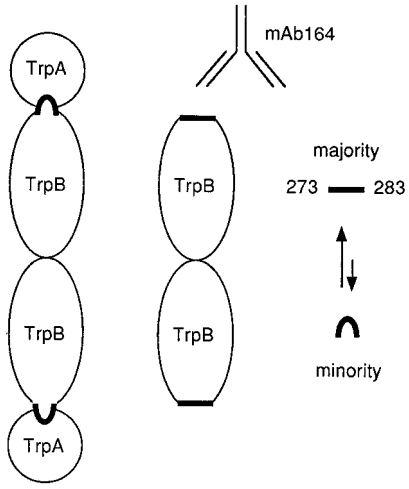

FIG. 1. **Interactions between mAb164 and antigens derived from tryptophan synthase.** mAb164 is directed against the $TrpB_2$ subunit of tryptophan synthase ($TrpA_2TrpB_2$). It recognizes the synthetic peptide P11, constituted of residues 273–283 of TrpB, with a strong affinity. The free molecules of P11 adopt an extended conformation in majority and a loop conformation in minority (18). The crystal structure of the free $TrpB_2$ is unknown. Residues 273–283 of TrpB are located in part within the interface between TrpA and TrpB in the crystal structure of $TrpA_2TrpB_2$.

measured the K_D for the interaction between the $TrpB_2$ variants and mAb164 by competition enzyme-linked immunosorbent assay. We then compared the effects of the mutations in each of the two contexts, $TrpB_2$ and MalE-P11, and found that these effects were strongly correlated.

MATERIALS AND METHODS

Strains and Vectors—The *E. coli* strain MD33 *(Δ(trpEA)2 tnaA2)* (21) and plasmid pTZ19R (22) have been described. Plasmid pαβTS21 comes from a laboratory collection.[2] It carries the *Sau3A* restriction fragment (681 base pairs) that contains the *E. coli tnaA* promoter, and the *Bgl*II-*Sal*I fragment (3328 base pairs) that contains the *E. coli trpB* and *trpA* genes, inserted into the *Bam*HI and *Eco*RI sites of pBR322 respectively (the cohesive ends of the *Eco*RI, *Bgl*II, and *Sal*I sites were filled in with dNTPs and Klenow polymerase prior to ligation) (21, 23).

Construction of Mutations in the trpB Gene—The *Bsu*36I-*Sal*I fragment of pαβTS21 that carries the *trpA* and *trpB* genes under control of the *tnaA* promoter, was recombined by ligation between the *Sma*I and *Sal*I sites of phagemid pTZ19R to give phagemid pPR2. The *Bsu*36I cohesive ends of pαβTS21 were filled in with dNTPs and Klenow polymerase prior to digestion with *Sal*I. The mutations of *trpB* were created by oligonucleotide site-directed mutagenesis, using the single-stranded DNA of phagemid pPR2 as template (24). The presence of the mutations in *trpB* was checked by DNA sequencing with the T7 sequencing kit (Amersham Pharmacia Biotech) and the oligonucleotide 5′-AATGAAACCAACGTCGGCCT-3′, which hybridizes upstream of the mutated region. Glucose (2% w/v) was added to the culture medium during all the genetic constructions to repress the *tnaA* promoter and prevent the expression of the *trpA* and *trpB* genes (25).

Production and Purification of the apo-$TrpB_2$ Proteins—Phagemid pPR2 and its mutants derivatives were introduced into strain MD33, which carries a deletion of the *trpA* and *trpB* genes, to express the wild type or mutant TrpA and $TrpB_2$ subunits. A preculture of the MD33 derivatives was grown overnight at 30 °C in LB medium, supplemented with 2% glucose and 100 μg/ml ampicillin. The cells were collected by centrifugation, resuspended in fresh medium without glucose (100 times the initial volume), and incubated at 30 °C until $A_{600\ nm} = 1.0$. The bacteria were harvested by centrifugation, and the pellet was frozen at −20 °C. The apo-form of the wild type or mutant $TrpB_2$ was purified by crystallization as described (26). The crystallized protein was kept in ammonium sulfate (37.5% saturation) at +4 °C. The purity of the protein was checked by electrophoresis through SDS-polyacrylamide gels and staining with Coomassie Blue. Before use, the purified preparation of apo-$TrpB_2$ was reactivated by a heat treatment and an overnight dialysis against a phosphate buffer containing 2-mercaptoethanol, as described (26). The affinities and enzymatic activities were measured within 48 h after reactivation. TrpA was purified as described (27). It was kept and used as a precipitate in ammonium sulfate. Monoclonal antibody mAb164 was produced by injection of the corresponding hybridoma cells in the peritoneum of mice and purified by chromatography on a DEAE-cellulose column as described (28, 29). The concentrations of purified proteins were measured with the Bio-Rad protein assay kit and bovine serum albumin as a standard for the $TrpB_2$ subunits and with $A_{280\ nm}$ and a molar extinction coefficient of 1.5 for mAb164 (30).

Equilibrium Dissociation Constants—The equilibrium dissociation constants, K_D, between antibody mAb164 and the $TrpB_2$ derivatives were measured by a competition enzyme-linked immunosorbent assay as described (20, 31). The measurements were performed at 25 °C in 0.02% bovine serum albumin, 2 mM Na_2EDTA, 0.1 M potassium phosphate, pH 7.8. In the mathematical treatment of the data, the total concentration of antigen was considered as twice the concentration of $TrpB_2$ because two molecules of mAb164 can bind one $TrpB_2$ subunit.[3]

Analysis of the Data and Structures—The energy of interaction ΔG between antibody mAb164 and a $TrpB_2$ derivative was calculated by

$$\Delta G = -\mathrm{RT}\ln(K_D), \quad \text{(Eq. 1)}$$

where R is the gas constant and $T = 298.15$ K. The variation ΔΔG of free energy when going from the wild type (wt) to a mutant (mut) $TrpB_2$ was calculated by the following equation.

$$\Delta\Delta G(\mathrm{wt, mut}) = \Delta G(\mathrm{wt}) - \Delta G(\mathrm{mut}). \quad \text{(Eq. 2)}$$

Its variation when going from a first mutant (mut_1) to a second mutant (mut_2) was calculated by the following equation.

$$\Delta\Delta G(\mathrm{mut_1, mut_2}) = \Delta G(\mathrm{mut_1}) - \Delta G(\mathrm{mut_2}) \quad \text{(Eq. 3)}$$

The effect of the less damaging mutation, mut_2, in the context of the more damaging mutation, mut_1, was calculated by the equation,

$$\begin{aligned}\Delta\Delta G_A(\mathrm{mut_1, mut_2}) &= \Delta\Delta G(\mathrm{wt, mut_1\text{-}mut_2}) - \Delta\Delta G(\mathrm{wt, mut_1}) \\ &= \Delta G(\mathrm{mut_1}) - \Delta G(\mathrm{mut_1\text{-}mut_2}) \quad \text{(Eq. 4)}\end{aligned}$$

where mut_1-mut_2 represents the double mutation. $\Delta\Delta G_B$, the coupling parameter between mutations mut_1 and mut_2, was calculated by the following equation.

$$\begin{aligned}\Delta\Delta G_B(\mathrm{mut_1, mut_2}) &= \Delta\Delta G(\mathrm{wt, mut_1\text{-}mut_2}) - \Delta\Delta G(\mathrm{wt, mut_1}) \\ &\quad - \Delta\Delta G(\mathrm{wt, mut_2}) \\ &= \Delta G(\mathrm{mut_1}) + \Delta G(\mathrm{mut_2}) - \Delta G(\mathrm{mut_1\text{-}mut_2}) \\ &\quad - \Delta G(\mathrm{wt}) \quad \text{(Eq. 5)}\end{aligned}$$

If measurements were a_i ($i = 1 \ldots n$), the S.E. on the sum of the a_i values was calculated from the S.E. on the individual a_i values by the following equation.

$$(\mathrm{SE}(\Sigma_i a_i))^2 = \Sigma_i(\mathrm{SE}(a_i))^2 \quad \text{(Eq. 6)}$$

We used the atomic coordinates of $TrpA_2TrpB_2(K^+)$, *i.e.* tryptophan synthase with a bound K^+ ion (PDB 1ttq; Ref. 17). The structure of $TrpA_2TrpB_2(K^+)$ was analyzed with the WHAT IF program (http://www.sander.embl-heidelberg.de/whatif/). The accessible surface area was calculated with the ACCESS routine, using a 1.4 Å radius probe. The contacts between residues and the potential for the formation of H-bonds were calculated with the ANACON and DIST routines. We used the extended Van der Waals radii (32) as described (33, 34).

RESULTS

Deletions of the Side Chains into Ala and Gly—Residues 273–283 of TrpB were first changed into Ala or Gly to delete their side chains (Table I). These changes showed that the side chains of four residues, Val^{276}, Ile^{278}, Tyr^{279}, and Phe^{280}, were predominant in the recognition of $TrpB_2$ by antibody mAb164 ($\Delta\Delta G \geq 2.8$ kcal·mol^{-1}). The side chains of Met^{282} and Lys^{283} were more weakly involved ($\Delta\Delta G = 1.2$ and 1.4 kcal·mol^{-1},

[2] C. Zetina and A. Chaffotte, unpublished data.

[3] M. P. Larvor, unpublished data.

TABLE I

Equilibrium constants and associated free energies for the dissociation between mAb164 and the $TrpB_2$ or MalE-P11 derivatives at 25 °C

The residues are numbered according to their positions in the sequence of TrpB. I278V, change in either TrpB or in the P11 moiety of MalE-P11 replacing the wild type side chain, Ile, at position 278 by Val; I278V/K283A, a double change; wt, wild-type protein; mut, a derivative carrying one of the changes of the first column; ΔASA, accessible surface area of the side-chain groups deleted by the mutation in the structural model of $TrpB_2$ (see text).

Mutation	$TrpB_2$ ΔASA	$TrpB_2$ K_D ± S.E.	$TrpB_2$ ΔG ± S.E.	$TrpB_2$ ΔΔG ± S.E.	MalE-P11 ΔΔG ± S.E.
	Å²	*nM*		*kcal·mol⁻¹*	
WT		0.13 ± 0.01	13.47 ± 0.07	0.0 ± 0.1	0.0 ± 0.1
H273A	64.2	0.08 ± 0.01	13.80 ± 0.10	−0.3 ± 0.1	−0.2 ± 0.1
R275A	83.5	0.25 ± 0.08	13.13 ± 0.19	0.3 ± 0.2	0.0 ± 0.1
V276A	36.8	50 ± 10	9.98 ± 0.13	3.5 ± 0.1	3.8 ± 0.2
I278A	61.5	361 ± 56	8.79 ± 0.10	4.7 ± 0.1	5.1 ± 0.6
I278V	45.6	0.33 ± 0.02	12.93 ± 0.03	0.5 ± 0.1	5.5 ± 0.1
Y279A	70.8	231 ± 9	9.07 ± 0.03	4.4 ± 0.1	4.6 ± 0.3
Y279F	23.1	0.21 ± 0.02	13.20 ± 0.05	0.3 ± 0.1	1.2 ± 0.6
Y279L	55.4	14 ± 2	10.73 ± 0.07	2.7 ± 0.1	2.4 ± 0.1
Y279P		79,400 ± 13,300	5.61 ± 0.11	7.9 ± 0.1	6.1 ± 0.3
F280A	2.1	0.28 ± 0.02	13.04 ± 0.05	0.4 ± 0.1	1.4 ± 0.2
F280G	7.6	16 ± 3	10.68 ± 0.09	2.8 ± 0.1	3.5 ± 0.1
F280P		365 ± 73	8.82 ± 0.13	4.7 ± 0.1	4.1 ± 0.1
G281A	0.0[a]	2.7 ± 0.3	11.70 ± 0.07	1.8 ± 0.1	2.5 ± 0.2
M282A	0.0	1.0 ± 0.2	12.31 ± 0.12	1.2 ± 0.1	1.9 ± 0.1
M282P		1320 ± 260	8.03 ± 0.12	5.4 ± 0.1	4.1 ± 0.2
K283A	58.6	1.5 ± 0.4	12.08 ± 0.15	1.4 ± 0.2	2.1 ± 0.3
V276A/K283A		3910 ± 930	7.42 ± 0.15	6.1 ± 0.2	5.9 ± 0.4
I278V/K283A		35 ± 7	10.20 ± 0.12	3.3 ± 0.1	3.7 ± 0.2
I278A/K283A		50,000 ± 8900	5.89 ± 0.11	7.6 ± 0.1	

[a] Accessible surface area of the $C_\alpha H_2$. The mean values and associated S.E. of K_D, $\Delta G = -RT\ln(K_D)$, and $\Delta\Delta G = \Delta G(\text{wt}) - \Delta G(\text{mut})$ in three independent experiments are given. The S.E. for ΔΔG was calculated as described under "Materials and Methods" (Equation 6). The values of ΔΔG and S.E. for the MalE-P11 derivatives are from Ref. 20.

respectively). The deletions of the side chains of His^{273} and Arg^{275} were nearly neutral. The three other residues were glycines and had no side chain. These results were compatible with the observation that the free energy of binding between proteins is generated by a small number of strong interactions and not by the accumulation of numerous weak contacts (35, 36).

Progressive Deletions of Side Chains—The four side chains that were predominant in the interaction between $TrpB_2$ and mAb164 were progressively deleted to test the contribution of their different groups to the binding of mAb164 (Table II). Val^{276} could only be changed to Ala. The changes of residue Ile^{278} into Val and Ala showed a weak contribution of its $C_\delta H_3$ group and a strong contribution of its $C_{\gamma 1}H_2$ and $C_{\gamma 2}H_3$. The changes of Tyr^{279} into Phe, Leu, and Ala showed a weak contribution of the hydroxyl group $O_\eta H$, an important contribution of the distal part of the aromatic cycle, and a slightly less important contribution of the proximal part of the cycle. The changes of Phe^{280} into Ala and Gly showed a weak contribution of its aromatic cycle and an important contribution of its $C_\beta H_2$. Thus, the $C_{\gamma 1}H_3$ or $C_{\gamma 2}H_3$ of Val276, the $C_{\gamma 1}H_2$ or $C_{\gamma 2}H_3$ of Ile278, the aromatic cycle of Tyr^{279}, and the $C_\beta H_2$ of Phe^{280} were the most important contributors to the interaction between $TrpB_2$ and mAb164.

Contribution of the Polypeptide Backbone—Residues Tyr^{279}–Met^{282} are located at the tip of a hairpin in the crystal structure of $TrpA_2TrpB_2$. We changed, individually, residues Tyr^{279}, Phe^{280}, and Met^{282} into prolines and Gly^{281} into alanine to probe the contribution of the polypeptide backbone of these four residues to the recognition of $TrpB_2$ by mAb164, according to a rationale previously described (Table I; Ref. 20). We compared the mutations into Pro with those into Ala to eliminate the effects of the side chains (Table III). The ΔΔG values for the changes from Ala into Pro (ΔΔG ≥ 3.4 kcal/mol) suggested that the recognition between $TrpB_2$ and mAb164 was incompatible with the conformational constraints that a Pro residue imposed at positions 279, 280, or 282. These ΔΔG values were higher than the energy of a H-bond, and therefore, the effects of the mutations into proline were not limited to the breaking of a H-bond involving the NH-peptide group (37). The destabilizing effect of mutation G281A on the interaction between $TrpB_2$ and mAb164 could be due to steric clashes between the mutant side chain and either residues of mAb164 or neighboring residues of $TrpB_2$.

TABLE II

Contributions of the side chain groups to the energy of interaction between mAb164 and either $TrpB_2$ or MalE-P11

F279A, change replacing a Phe side chain at position 279 by Ala. The contribution of the side chain groups deleted by F279A was calculated by $\Delta\Delta G = \Delta G(\text{Y279F}) - \Delta G(\text{Y279A})$; its associated S.E. value was calculated from the S.E. values on ΔG(Y279F) and ΔG(Y279A) as described under "Materials and Methods" (Equation 6). The values of ΔG ± S.E. for the $TrpB_2$ derivatives and the definition of ΔASA are given in Table I. The values of ΔΔG ± S.E. for the MalE-P11 derivatives are from Ref. 20.

Mutation	$TrpB_2$ ΔASA	$TrpB_2$ ΔΔG ± S.E.	MalE-P11 ΔΔG ± S.E.
	Å²	*kcal·mol⁻¹*	
I278V	45.6	0.54 ± 0.07	5.5 ± 0.1
V278A	15.9	4.14 ± 0.10	−0.4 ± 0.6
Y279F	23.1	0.27 ± 0.09	1.2 ± 0.6
F279L	32.3	2.47 ± 0.09	1.2 ± 0.6
F279A	47.7	4.13 ± 0.06	3.4 ± 0.7
L279A	15.4	1.66 ± 0.08	2.2 ± 0.3
F280A	2.1	0.43 ± 0.08	1.4 ± 0.2
A280G	5.5	2.36 ± 0.10	2.1 ± 0.1

TABLE III

Variations in the energy of interaction between mAb164 and either $TrpB_2$ or MalE-P11 for changes from Ala into Pro

Notations are as in Table II. The values of ΔG ± S.E. for the $TrpB_2$ derivatives are given in Table I. The values of ΔΔG ± S.E. for the MalE-P11 derivatives are from Ref. 20.

Mutation	$TrpB_2$ ΔΔG ± S.E.	MalE-P11 ΔΔG ± S.E.
	kcal·mol⁻¹	
A279P	3.4 ± 0.2	1.5 ± 0.3
A280P	4.2 ± 0.1	2.7 ± 0.2
A282P	4.3 ± 0.2	2.2 ± 0.2

Tertiary Interactions within Residues 273–283—To test the existence of long range tertiary interactions between residues

TABLE IV
Coupling energies between mutations of the antigen

mut_1 and mut_2, mutations of $TrpB_2$ or MalE-P11; mut_1, more damaging mutation for the interaction with mAb164; mut_2, less damaging mutation; $\Delta\Delta G_2 = \Delta\Delta G(wt, mut_2)$, effect of mut_2 in the context of the wild type; $\Delta\Delta G_A$, effect of mut_2 in the context of mut_1; $\Delta\Delta G_B$, coupling energy between mut_1 and mut_2. The values of $\Delta\Delta G_2 \pm$ S.E. were taken from Table I; those of $\Delta\Delta G_A$, $\Delta\Delta G_B$ and their associated S.E. values were calculated as described under "Materials and Methods" (Equations 4–6) and Ref. 43.

mut_1	mut_2	Context	$\Delta\Delta G_2 \pm$ S.E.	$\Delta\Delta G_A \pm$ S.E.	$\Delta\Delta G_B \pm$ S.E.
				kcal·mol^{-1}	
V276A	K283A	$TrpB_2$	1.4 ± 0.2	2.6 ± 0.2	1.2 ± 0.3
K283A	I278V	$TrpB_2$	0.5 ± 0.1	1.9 ± 0.2	1.3 ± 0.2
I278A	K283A	$TrpB_2$	1.4 ± 0.2	2.9 ± 0.1	1.5 ± 0.2
V276A	K283A	MalE-P11	2.1 ± 0.3	2.0 ± 0.4	−0.1 ± 0.5
I278V	K283A	MalE-P11	2.1 ± 0.3	−1.8 ± 0.2	−4.0 ± 0.3

273–283 of $TrpB_2$ and the importance of these interactions for the recognition of $TrpB_2$ by mAb164, we constructed three double changes in $TrpB_2$ (Table I). The energy of interaction between the two mutated residues, $\Delta\Delta G_B$, was positive in the three studied cases (from 1.2 to 1.5 kcal·mol^{-1}; Table IV). In other words, the loss in free energy of interaction that resulted from the double mutation was higher than the sum of the losses for the two single mutations. Moreover, the less deleterious of the two mutations was more destabilizing in the context of the more deleterious mutation than in the context of the wild type (Table IV). Thus, the effects of the two mutations were synergistic.

Activities of the Mutant $TrpB_2$—The $TrpB_2$ subunit catalyzes the condensation of indole and serine into tryptophan, and this tryptophan synthase activity is strongly enhanced in the $TrpA_2TrpB_2$ complex (38). We assayed this activity at 25 °C, either in the presence of an excess of TrpA (>5-fold) or in the absence of TrpA, and we monitored the reaction with $A_{289\ nm}$ as described (39). The specific activities of the mutant $TrpB_2$ subunits were either high (>50% that of the wild type) or strongly increased by the addition of TrpA (by a factor at least equal to that of the wild type, *i.e.* >28-fold). These results showed that all the mutant derivatives of $TrpB_2$ were at least partially functional and that they had a correct global fold.[4]

DISCUSSION

Similarities in the Recognition of the Integrated and Isolated Versions of the Epitope—Generally, the ΔΔG values for the mutants of $TrpB_2$ were close to those for the mutants of MalE-P11, with an average difference between these values equal to −0.35 ± 0.31 (average ± S.E.) for the whole set of mutations, and −0.10 ± 0.18 if mutation I278V was excluded. The values of ΔΔG for $TrpB_2$ and MalE-P11 were strongly correlated, with a coefficient of correlation equal to 0.82 for the whole set of mutations and to 0.96 without mutation I278V (Fig. 2). These comparisons and correlations between mutations of which the natures were very diverse and the associated ΔΔG covered a wide range of values showed that mAb164 recognized the integrated and isolated forms of residues 273–283 according to the same global molecular mechanism.

Differences in the Recognitions of the Two Versions of the Epitope—The energy of interaction ΔG between mAb164 and the antigen was equal to 13.5 ± 0.1 kcal/mol for $TrpB_2$ and to 11.3 ± 0.1 kcal/mol for MalE-P11 (Table I; Ref. 20). Similar differences in the energies of interaction with mAb164 have been reported for $TrpB_2$ and an isolated synthetic peptide P11 (14, 15). The difference between the ΔG values for the integrated and isolated forms of the antigen, 2.2 kcal/mol, could have several causes. Some neighboring residues of $TrpB_2$, located outside the segment 273–283, could be directly involved in the interaction with mAb164. Alternatively, neighboring residues could be indirectly involved in the interaction, by stabilizing the recognized conformation of residues 273–283.

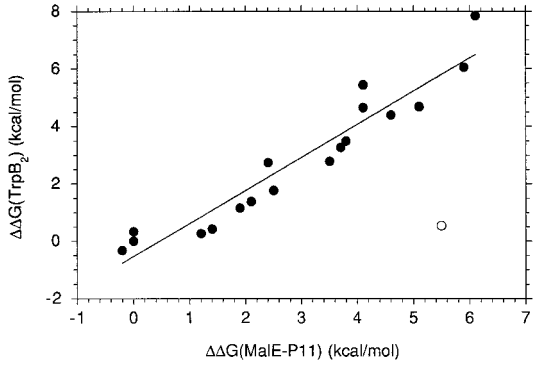

FIG. 2. **Correlation between the effects of the mutations on the interaction with mAb164 in the $TrpB_2$ context and in the MalE-P11 context.** The values of ΔΔG are from Table I. The value of ΔΔG for mutation I278V (*open circle*) was excluded from the correlation. The Pearson *R* coefficient was equal to 0.96.

The sum of the ΔΔG values for the mutations that cut the side chains into Ala was equal to 15.6 kcal/mol for $TrpB_2$ and 18.7 kcal/mol for MalE-P11 (Table I). These sums were higher than the ΔG values for the wild type antigens, 13.5 and 11.3 kcal/mol, respectively. The energy surplus is generally attributed to some dependence between the effects of the mutations. This surplus was lower for $TrpB_2$, 2.1 kcal/mol, than for MalE-P11, 7.4 kcal/mol. This comparison suggested that the indirect effects of the mutations into Ala, *i.e.* through conformational changes of neighboring residues, were smaller for the integrated than for the isolated version of residues 273–283.

The structural environment of residues 273–283 comprised only the other residues of this protein segment in the isolated version of the epitope and the whole $TrpB_2$ in its integrated version. Therefore, a mutation in segment 273–283 could have a larger effect on the conformation of the neighboring residues and, indirectly, on the recognition by mAb164 when this segment was isolated than when it was integrated. We found that mutations F280A, G281A, M282A, and K283A, which were of secondary importance, were more destabilizing in the context of MalE-P11 than in the context of $TrpB_2$. These findings suggested that the side chains of the four corresponding residues, Phe^{280}–Lys^{283}, played an indirect, conformational role in the recognition by mAb164.

Val^{276}, Ile^{278}, and Tyr^{279} were the three most important residues for the recognition of the antigen by mAb164. The global contributions of their side chains to the energy of interaction were the same in the two contexts, MalE-P11 and $TrpB_2$ (Table I). Nevertheless, the distribution of these contributions within the side chains could be different in the two contexts (Table II). Mutation I278V had less effect and V278A more effect in the $TrpB_2$ context than in the MalE-P11 context. Therefore, the contribution of the Ile^{278} side chain was redistributed toward its $C_{\gamma 1}H_2$ and $C_{\gamma 2}H_3$ groups in the $TrpB_2$ context. Mutations Y279F and L279A had less effect and F279L more effect in the $TrpB_2$ context than in the MalE-P11 context. Therefore, the contribution of the Tyr^{279} side chain was redistributed toward the distal half of the aromatic cycle in the $TrpB_2$ context. These comparisons showed some adaptation of the interaction between antibody mAb164 and either $TrpB_2$ or peptide P11 at the level of each residue.

The effects of mutations A279P, A280P, and A282P were

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stronger in the $TrpB_2$ context than in the MalE-P11 one. This difference could be due to the fact that the N- and C-terminal ends of segment 273–283 were linked and fixed in the context of $TrpB_2$ so that any variation in the (ϕ,ψ) dihedral angles of one residue (as introduced by a change into Pro) necessarily introduced compensatory changes of angles and thus conformational changes elsewhere in the segment. In contrast, the C-terminal end of segment 273–283 was free in the MalE-P11 context, and therefore, this segment could rotate around the peptide bond that preceded His^{273} in response to a change in the (ϕ,ψ) angles of a residue.

The double mutations I278V/K283A and V276A/K283A had the same effects on the interaction with mAb164 in the two structural contexts. In the MalE-P11 context, mutation I278V has a strong effect and V278A a weak one (Table I). Moreover, I278V and K283A have strongly antagonistic effects, with a compensatory effect of K283A on the I278V effect. We have deduced from these effects that I278V induces a conformational change of peptide P11, that this change leads to a complete loss of the interaction energy between the side chain of Ile278 and mAb164, and that it is compensated by mutation K283A. In the same context, V276A and K283A have purely additive effects (Table IV; Ref. 20). In the $TrpB_2$ context, mutations K283A on the one hand, and either V276A, I278V, or I278A on the other hand, had synergistic effects (Table IV). These synergies could have two causes. The double mutations could induce conformational changes of residues 273–283 that were unfavorable for the interaction with mAb164. Alternatively, the synergies could be due to an anticooperativity between Lys^{283} on the one hand and Val^{276} and Ile^{278} on the other hand for the binding of mAb164. In other words, each of the side-chain would prevent an optimal interaction between the other side chain and mAb164. In the two contexts, MalE-P11 and $TrpB_2$, the results suggested a proximity of the side chains of Ile^{278} and Lys^{283} in the antigen, because the effect of a change in one of the two residues depended on the side-chain of the other residue.

Implications for the Conformation of the Integrated Epitope—Antibody mAb164 is directed against the free and apo-form of protein $TrpB_2$ and we studied the variants of $TrpB_2$ in this form. A priori, the conformation of $TrpB_2$ that is recognized by mAb164 could differ from the structure that this protein adopts in the crystals of $TrpA_2TrpB_2$ for the following reasons. The crystallized complex contains the holoform of $TrpB_2$. The $TrpB_2$ subunit undergoes a conformational change when it associates with TrpA (38). Several residues of segment 273–283 belong to the interface between TrpA and $TrpB_2$. The conformations of residues Tyr^{279} and Phe^{280} depend on the structural context and are different when a K^+ or Na^+ ion is bound to $TrpB_2$ (17).

The solvent-accessible surface area of residues 273–283 of TrpB partitions as follows in the crystal structure of $TrpA_2TrpB_2(K^+)$: 357 $Å^2$ are accessible from the outside of the tetramer, 228 $Å^2$ are buried in the interface with TrpA, and 1002 $Å^2$ are buried in the interface with the remainder of $TrpB_2$. Therefore, residues 273–283 make more extensive contacts with the remainder of $TrpB_2$ than with TrpA. This result is also valid for each of residues 273–283, taken individually, except for Ile^{278}, the solvent-accessible surface area of which is more buried by TrpA (for 93 $Å^2$) than by $TrpB_2$ (42 $Å^2$). These area values show that residues 273–283 of TrpB are well anchored at the surface of $TrpB_2$ and suggest that their structures in the free form of $TrpB_2$ and in its complex with TrpA are close. Therefore, we constructed a structural model of the free form of $TrpB_2$ simply by removing the atoms of TrpA in the structure of $TrpA_2TrpB_2$.

Three residues, Val^{276}, Ile^{278}, and Tyr^{279}, were of primary importance in the recognition of $TrpB_2$ by mAb164. The side chains of these three residues and, in particular, their active groups were strongly exposed to the solvent in the structural model of $TrpB_2$ (Tables I and II). The aromatic cycle of Phe^{280} contributed weakly to the recognition by mAb164, whereas its $C_\beta H_2$ group contributed strongly. $C_\beta H_2$ was the only group of Phe^{280} that was accessible to the solvent, at least partially. Three residues, Gly^{281}, Met^{282}, and Lys^{283}, were of secondary importance in the recognition. Their side chains were little exposed to the solvent or even totally buried. In particular, the $C_\beta H_2$ to $C_\delta H_2$ groups of Lys^{283} were fully buried in the model of $TrpB_2$. The side-chain groups that were of secondary importance formed contacts with the side-chain groups that were of primary importance: the aromatic cycle of Phe^{280} with Tyr^{279}, Met^{282} with the $C_\beta H_2$ of Phe^{280}, and Lys^{283} with Val^{276} and Ile^{278} (Table V). These comparisons of the solvent-accessible surface areas and of the contacts between residues with our results of mutagenesis on $TrpB_2$ suggested that the dissociation of TrpA and $TrpB_2$ did not induce an important change in the structure of residues 276–283 of TrpB. They also strengthened the conclusion that residues 280–283 could have an indirect conformational role in the recognition of $TrpB_2$ and MalE-P11 by mAb164.

TABLE V
Contacts potentially altered by the mutations in $TrpB_2$

Only the contacts and the potential H-bonds with other residues of $TrpB_2$ are indicated. They were calculated from the crystal structure of $TrpA_2TrpB_2(K^+)$, as described under "Materials and Methods." B, contact with a backbone atom; S, contact with a side-chain atom; H-bond, potential H-bond.

Mutation	Potential contacts
H273A	Ile^{262} (S)
R275A	Gln^{288} (S), Thr^{289} (B, H-bond), Ala^{290} (B)
V276A	Phe^{12} (S), Arg^{275} (B), Gly^{277} (B), Lys^{283} (S), Ala^{284} (B), Pro^{285} (S)
I278V	Tyr^{16} (S)
V278A	Tyr^{16} (S), Gly^{277} (B), Tyr^{279} (B), Met^{282} (B), Lys^{283} (S)
F279L	Phe^{280} (S), Ile^{294} (S), HOH^{473}
L279A	Phe^{280} (B, S), Ile^{294} (S), HOH^{435}, HOH^{473}
F280A	Lys^{167} (B, S), Tyr^{279} (B, S), Phe^{306} (S), Pro^{307} (S)
A280G	Tyr^{279} (B), Gly^{281} (B), Met^{282} (S)
G281A	Tyr^{16} (B), Pro^{194} (S), Met^{282} (S), HOH^{434}
M282A	Ala^{192} (B, S), Gly^{193} (B), Tyr^{279} (B), Phe^{280} (S), Gly^{281} (B), Lys^{283} (B), Ala^{284} (S), Phe^{306} (S), Ser^{308} (S), Pro^{311} (S)
K283A	Glu^{11} (B, H-bond; S), Tyr^{16} (S), Val^{276} (S), Ile^{278} (S), Ala^{284} (B)

Residues 275–286 of TrpB adopt a hairpin conformation in the crystal structure of $TrpA_2TrpB_2(K^+)$. More specifically, residues Ile^{278}–Met^{282} form a type $\beta_{E\gamma\gamma}$ double turn in a 2:2 hairpin (40). The structure is compatible with the existence of two hydrogen bonds, between the NH and CO peptide groups of Tyr^{279} and Met^{282}. These observations are valid not only for the structure with the K^+ ion (Fig. 3) (this work) but also for the structure with the Na^+ ion (20). Proline adopts well defined (ϕ,ψ) dihedral angles and therefore introduces constraints on the conformation of the polypeptide backbone (41). We measured the (ϕ,ψ) angles of residues Tyr^{279}, Phe^{280}, and Met^{282} in the structure of $TrpA_2TrpB_2(K^+)$ and calculated the distance between each of these residues and the closest typical residue of *trans*-Pro in the Ramachandran plan, as described (20). We found that this distance was equal to 101° for Tyr^{279}, 171° for Phe^{280}, and 77° for Met^{282}. Therefore, these distances were large. Compatibly, the high values of $\Delta\Delta G$ that were associated with mutations A279P, A280P, and A282P ($\geq$3.4 kcal/mol) showed that the effects of the mutations into Pro were not limited to the breaking of the H-bonds involving the NH peptide groups and indicated that they had a conformational component. Gly281 occupies the fourth position of the $\beta_{E\gamma\gamma}$ turn,

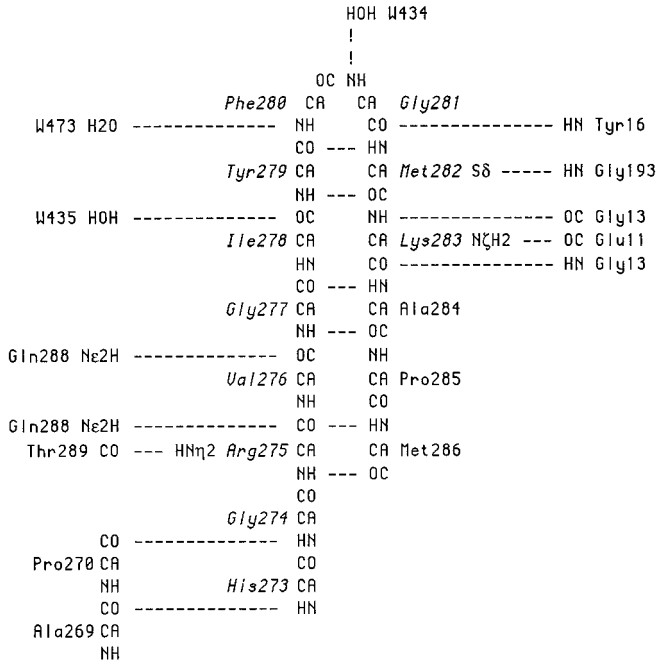

FIG. 3. **Schematic structure of residues 273–283 of TrpB in the crystal structure of $TrpA_2TrpB_2(K^+)$.** The potential H-bonds between residues 273–283 of TrpB (*italics*) and either residues of TrpB or water molecules are indicated by *dashed lines*. They were calculated as described under "Materials and Methods."

formed by residues Ile^{278} to Met^{282}, in the structure of $TrpA_2TrpB_2$ and there is a strong preference for a Gly residue at this position of a $\beta_{E\gamma\gamma}$ turn (40). Compatibly, mutation G281A had a strong destabilizing effect on the interaction between $TrpB_2$ and mAb164.

The structure of $TrpA_2TrpB_2$ shows contacts between the side chain of Lys^{283} and those of Val^{276} and Ile^{278} (Table V). Compatibly, the comparison of the effects of the double mutations I278V/K283A, I278A/K283A, and V276A/K283A with those of the single mutations showed that interactions between the side chains of Lys^{283} and those of Ile^{278} and Val^{276} were involved in the recognition between $TrpB_2$ and mAb164. They suggested that these side chains were close in space and therefore that residues 276–283 were recognized in a loop conformation.

Comparison with Other Systems and Conclusions—Several crystal structures of complexes between oligopeptides and antibody fragments have been determined and compared with the structures of the native proteins from which the peptides were derived, to better understand the structural bases of cross-reactivity. These comparisons have shown a great variety of situations. The structure of the isolated version of the peptide, bound to the antibody, can be similar to the structure of its integrated version, in the free native protein (10, 12). This situation generally corresponds to a strong cross-reactivity. Only a portion of the peptide can have similar structures in its bound isolated version and its free integrated version (8, 11). The two structures can be widely different (7, 9, 13). In this last situation, the difference in structure either allows us to explain the large difference (up to 1000-fold) in affinity for the antibody between the isolated peptide and the native protein or is attributed to a conformational change of the native protein, induced by the binding of the antibody or by the experimental conditions in the cross-reactivity assay.

We used mutations of residues 273–283 of TrpB, in the $TrpB_2$ context and in the MalE-P11 context (which is equivalent to the synthetic peptide P11), as local probes of their interactions with mAb164 and of their structures. We reached the following conclusions (this work and Refs. 19 and 20). Residues 273–283 are recognized through the same global interactions in the $TrpB_2$ context and in the MalE-P11 context. However, there is an adaptation of these interactions at the level of some side chains. The recognition depends mainly on four hydrophobic residues. Buried residues indirectly affect the recognition by the antibody. Residues 273–283 are recognized in a loop conformation, close to their structure in the crystals of the heterotetramer $TrpA_2TrpB_2$. Therefore, the dissociation of TrpA and $TrpB_2$ does not strongly perturb the structure of these residues, even though they are partly located in the interface between the two subunits. The antibody selects the molecules of peptide P11 that have a conformation similar to that of residues 273–283 in the structure of $TrpB_2$, which was used as immunogen. Thus, a mutational approach can give access to a description and a precise comparison of the recognition mechanisms for the isolated and integrated versions of a given epitope, in the absence of the crystal structures of the complexes. To our knowledge, only one other study of cross-reactivity by a mutational approach has been reported (42). However, only three single mutations were constructed in that case, and the affinities were determined only in a semi-quantitative way.

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