Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5783–5787, June 1995
Biochemistry

A molecular sensor system based on genetically engineered alkaline phosphatase

(protein engineering/signaling protein/modulation/allosteric regulation)

CATHERINE A. BRENNAN, KRIS CHRISTIANSON*, MARI ANNE LA FLEUR, AND WLODEK MANDECKI†

Virus Discovery, D-90D, Abbott Laboratories, 1401 Sheridan Road, North Chicago, IL 60064-4000

Communicated by Marvin H. Caruthers, University of Colorado, Boulder, CO, February 21, 1995 (received for review November 23, 1994)

ABSTRACT **Binding and signaling proteins based on *Escherichia coli* alkaline phosphatase (AP; EC 3.1.3.1) were designed for the detection of antibodies. Hybrid proteins were constructed by using wild-type AP and point mutants of AP [Asp-101 → Ser (D101S) and Asp-153 → Gly (D153G)]. The binding function of the hybrid proteins is provided by a peptide epitope inserted between amino acids 407 and 408 in AP. Binding of anti-epitope antibodies to the hybrid proteins modulates the enzyme activity of the hybrids; upon antibody binding, enzyme activity can increase to as much as 300% of the level of activity in the absence of antibody or can decrease as much as 40%, depending on the presence or absence of the point mutations in AP. The fact that modulation is altered from inhibition to activation by single amino acid changes in the active site of AP suggests that the mechanism for modulation is due to structural alterations upon antibody binding. Modulation is a general phenomenon. The properties of the system are demonstrated by using two epitopes, one from the V3 loop of human immunodeficiency virus type 1 gp120 protein and one from hepatitis C virus core protein, and corresponding monoclonal antibodies. The trend of modulation is consistent for all hybrids; those in wild-type AP are inhibited by antibody, while those in the AP mutants are activated by antibody. This demonstrates that modulation of enzyme activity of the AP–epitope hybrid proteins is not specific to either a particular epitope sequence or a particular antibody–epitope combination.**

Regulation of enzyme activity by binding a factor, small molecule, or protein is common (signal transduction, allosteric enzymes). While systems of this type are abundant in nature, construction of an artificial system presents a challenge; the surface of the enzyme must be modified to create a binding site for another protein, yet at the same time the catalytic activity of the enzyme must be maintained. In addition, the binding event must result in structural changes that alter the catalytic activity of the engineered enzyme.

Here, we report a molecular sensor system based on the regulation of the enzyme activity of genetically engineered *Escherichia coli* alkaline phosphatase (AP; EC 3.1.3.1). A known linear peptide epitope sequence is introduced onto the surface of AP in a defined location, thus creating a binding site for an antibody. We show that the enzymatic activity of such a hybrid enzyme is reduced or increased upon binding of an antibody specific for the epitope. The direction of activity change depends on the presence or absence of point mutations in the vicinity of the AP active site.

AP is a nonspecific phosphomonoesterase with a high catalytic turnover number (k_{cat}). The AP homodimer (M_r = 94,000) contains 4 Zn^{2+} and 2 Mg^{2+} metal ions (1). Several point mutations in the vicinity of the active site have been shown to alter the enzymatic activity of AP. Alteration of the amino acid residue Asp-101 → Ser (D101S) results in a dramatic increase in k_{cat} compared with wild-type AP (2). In the D101S mutant, the increased k_{cat} of the D101S protein is due to faster release of the phosphate product which is caused by structural changes in the active site that increase the flexibility of residues 101 and Arg-166 (3). The residue Asp-153, in the active site of AP, has a role in stabilizing Mg^{2+} binding (4). The mutants D153H (Asp-153 → His) (4), D153A (Asp-153 → Ala) (5), and D153G (Asp-153 → Gly) (W.M., K.C., and C.B., unpublished data) all show weaker affinity for Mg^{2+}. The activity of these mutants is affected by the state of metal binding; Mg-dependent increases in k_{cat} are likely due to stabilization of the active conformation of the enzymes. In addition, the binding affinity of phosphate is affected by these mutations; tighter binding of phosphate by the D153H mutant lowers k_{cat} (4, 6), while weaker affinity for phosphate by the D153A mutant increases k_{cat} (5).

In previous work (7), we documented an example of inhibition of the enzymatic activity of a hybrid AP that contains an insert of 13 aa from the V3 loop of human immunodeficiency virus type 1 (HIV 1) gp120 upon binding of an anti-gp120 monoclonal antibody (mAb). The epitope was inserted at the gene level between aa 407 and 408 in AP. The enzymatic activity of this hybrid protein, API1, is inhibited 40–50% when the anti-gp120 mAb binds to the inserted peptide (7). Here, we find that the introduction of the point mutations D101S and D153G into API1 results in a shift from inhibition of enzyme activity upon antibody binding to an increase in activity upon antibody binding. This change from inhibition to activation, caused by single amino acid changes in AP, is consistent with a mechanism of modulation based on structural alteration.

To determine the general nature of modulation of AP–epitope hybrid proteins, a 15-aa peptide epitope from the hepatitis C virus (HCV) core protein (aa 50–64 in the core) (8) was inserted between aa 407 and 408 in wild-type AP and the D101S and D153G AP mutants. In the presence of several anti-HCV core mAbs, a consistent pattern of modulation is observed. The hybrid protein with the HCV epitope in wild-type AP is inhibited by the mAbs, whereas the hybrid proteins containing the D101S and D153G point mutations are activated by the mAbs. These results demonstrate that modulation by antibodies is not specific to a particular amino acid sequence inserted into AP nor is it specific to a particular epitope–antibody combination. The consistent pattern of inhibition or activation, depending on the AP background of the hybrid protein, suggests there may be a common mechanism for inhibition and a common mechanism for activation.

MATERIALS AND METHODS

Plasmid Constructions. The AP–epitope hybrid proteins were generated by modifying plasmids that contain a synthetic

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AP, alkaline phosphatase; HIV, human immunodeficiency virus; HCV, hepatitis C virus; mAb, monoclonal antibody; NS, nonspecific.

*Present address: BioNebraska, 3820 NW 46th Street, Lincoln, NE 68524.
†To whom reprint requests should be addressed.

phoA gene encoding wild-type alkaline phosphatase (9), a mutant *phoA* gene that has Asp-101 changed to Ser (D101S; in plasmid pMA115puc; ref. 2), or a mutant *phoA* gene that has Asp-153 changed to Gly (D153G; pDG201). The genes for the AP–epitope proteins were constructed by restriction-fragment replacement by using synthetic DNA fragments or restriction fragments isolated from plasmids that encode the desired amino acid sequence. All constructs were characterized by restriction analysis and DNA sequencing.

Protein Purification. The AP hybrid proteins were expressed and purified as described (7). Final protein preparations were stored at 4°C in 50 mM Tris acetate, pH 8.0, 1 mM $MgCl_2$/0.01 mM $ZnCl_2$/0.1% sodium azide. Protein concentrations and purity were determined as described (7); all proteins were >95% pure with the exception of API2, which was >90% pure.

Enzyme Assays. AP activity was assayed in solution at room temperature by monitoring the conversion of *p*-nitrophenyl phosphate (PNPP) to *p*-nitrophenol (PNP) as described (7). The effect of mAbs on the enzyme activity of hybrid proteins was determined by incubating AP hybrid protein (5 nM monomer) with various concentrations of mAb in 50 mM Tris·HCl, pH 8.0/1 mM $MgCl_2$/0.1 mg of bovine serum albumin per ml at room temperature for 5–10 min; PNPP (2 mM final concentration) was then added, and the rate of substrate hydrolysis was determined. The modulation of enzyme activity by mAb was determined by dividing the initial rate of substrate hydrolysis in the presence of antibody by the initial rate in the absence of antibody.

mAbs and Peptides. The anti-gp120 mAb was obtained from American Biotechnologies (Cambridge, MA). Anti-HCV core antibodies were obtained from the Monoclonal Antibody Development group at Abbott Laboratories. All the antibodies were dialyzed against Tris-buffered saline (50 mM Tris·HCl, pH 7.5/150 mM NaCl) by using a Pierce microdialyzer. Protein concentrations were determined after dialysis by using a Pierce BCA assay kit with bovine serum albumin as the standard.

The following peptides were used for peptide competition experiments. HIV-1 gp120 peptide (244010; Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys) was obtained from American Biotechnologies. An HCV peptide (Gln-Pro-Arg-Gly-Arg-Arg-Gln-Pro-Ile-Pro) was provided by K. Jaffe (Abbott), and a nonspecific (NS) peptide (Arg-Tyr-Ser-Val-Ile-Leu-Leu-Asp-Thr-Leu-Leu-Gly-Arg-Met-Leu) was provided by T. Leung (Abbott). The underlined sequences indicate the portion of each peptide that corresponds to the entire or partial peptide sequence that was inserted into the AP–gp120 or AP–HCV core hybrid proteins.

Biacore Procedures. Binding constants for the AP–epitope hybrid proteins and mAbs were determined by using a Biacore instrument (Pharmacia Biosensor). Rabbit anti-mouse Fcγ antibody (Ramfc; 50 μg/ml in 10 mM sodium acetate, pH 5; Pharmacia Biosensor) was immobilized to sensor chip CM5 (Pharmacia Biosensor) by using an amine-coupling kit (Pharmacia Biosensor) according to the manufacturer's instructions. For each kinetic run, mAb [25 μl at 30 μg/ml in phosphate-buffered saline (PBS; 2.7 mM KCl/138 mM NaCl/1.2 mM KH_2PO_4/8 mM Na_2HPO_4, pH 7)] was injected and captured on the sensor chip by the Ramfc, and then 15 μl of the AP–epitope hybrid protein at various concentrations in PBS was passed over the chip. The runs were performed at room temperature at a flow rate of 5 μl/min. Control experiments were performed to demonstrate the lack of nonspecific binding by the AP–epitope hybrid protein to the sensor chip or Ramfc.

RESULTS

Introduction of Point Mutations Results in Activation of Enzyme Activity upon Antibody Binding. Two point mutations were introduced into API1. The mutant D101S-I1 contains the Asp-101 → Ser (D101S) mutation in API1; mutant D153G-I1 contains the Asp-153 → Gly (D153G) mutation in API1. The rationale behind introducing these point mutations into API1 was as follows: if modulation of enzyme activity by antibody binding was due to conformational changes in or near the active site of AP, then these point mutations that alter active site residues and result in less ordered, more flexible active sites may enhance modulation—i.e., a less rigidly structured active site may be more easily distorted. In addition, these point mutations that increase the k_{cat} of wild-type AP may also increase the k_{cat} of the hybrid proteins.

Fig. 1 shows the effect of anti-gp120 mAb on the enzyme activity of API1, D101S, D153G, D101S-I1, and D153G-I1 proteins. The activity of the parent proteins D101S, D153G, and wild type (not shown) that do not contain the gp120 epitope is not affected by the presence of anti-gp120 mAb. Consistent with previous results (7), API1 is inhibited 40% by the presence of anti-gp120 mAb. Surprisingly, the introduction of single point mutations into AP results in a shift from inhibition to activation of enzyme activity upon antibody binding. D101S-I1 is activated to 180% and D153G-I1 is activated to 260% of the activity in the absence of antibody.

As with API1, the modulation of D101S-I1 and D153G-I1 by anti-gp120 mAb is specific for an antibody to the epitope insert; an unrelated anti-carcinoembryonic antigen mAb, H19C91 (10), does not either activate or inhibit enzyme activity (Table 1). In addition, the presence of free peptide 244010, which contains the inserted epitope sequence, blocks activation of the hybrid proteins by the anti-gp120 mAb (Table 1). An unrelated peptide (NS peptide) inhibited all of the enzymes by 10–35% but did not block activation of D153G-I1 and D101S-I1 or inhibition of API1 by the anti-gp120 mAb.

AP-HCV Core Hybrid Proteins Are Modulated by Anti-Core mAbs. To determine if the phenomenon of modulation of AP–epitope proteins by an antibody is specific to the particular epitope inserted into AP or a specific antibody, hybrid proteins were constructed that contain an epitope from the HCV core protein. A sequence of 15 aa from aa 50 to 64 in HCV core (ref. 8; Arg-Lys-Thr-Ser-Glu-Arg-Ser-Gln-Pro-Arg-Gly-Arg-Arg-Gln-Pro) was inserted between aa 407 and 408 in wild-type AP and in the AP mutants D101S and D153G to generate API2, D101S-I2, and D153G-I2, respectively.

Six anti-HCV core mAbs were tested for modulation of API2, D101S-I2, and D153G-I2 enzyme activity (Table 2). The antibodies have no effect on the enzyme activity of wild-type AP, D101S, and D153G (with the exception of a 20% activation of D153G by antibody 14-153-462). All the mAbs, except one, cause a small decrease in enzyme activity of API2 (15–

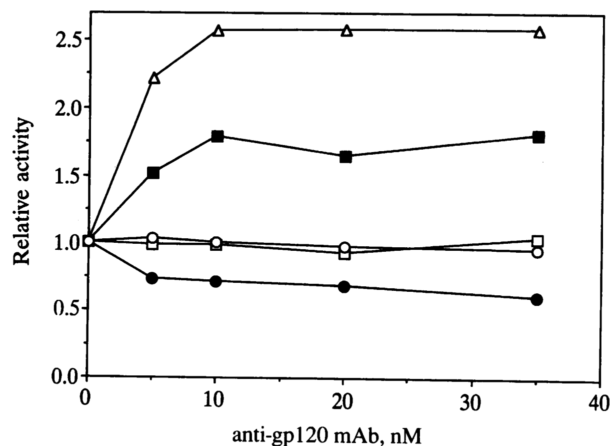

FIG. 1. Modulation of AP–epitope hybrid proteins as a function of anti-gp120 mAb concentration. Activity is expressed relative to the initial rate of hydrolysis of *p*-nitrophenyl phosphate in the absence of mAb. □, D101S; ○, D153G; ●, API1; ■, D101S-I1; and △, D153G-I1.

Table 1. Activation of AP–gp120 hybrid proteins is specific

	Relative enzyme activity				
mAb/peptide	API1	D101S-I1	D101S	D153G-I1	D153G
No mAb/no peptide	1.0	1.0	1.0	1.0	1.0
Anti-gp120 mAb/no peptide	**0.56**	**1.81**	0.99	**2.66**	1.0
H19C91 mAb/no peptide	0.99	0.98	0.88	1.11	0.99
No mAb/gp120 peptide	1.04	**1.21**	1.01	0.92	1.0
Anti-gp120 mAb/gp120 peptide	1.07	**1.26**	0.99	1.03	0.99
No mAb/NS peptide	0.88	**0.79**	**0.82**	**0.65**	**0.74**
Anti-gp120 mAb/NS peptide	**0.55**	**1.57**	**0.84**	**2.15**	**0.80**

The hybrid proteins (5 nM) were assayed in the absence or presence of antibody (35 nM) and/or peptide (4 μM). Enzyme activity is expressed relative to the activity of the hybrid protein in absence of antibody and peptide. Values of enzyme activity that differ from the control by more than 15% are shown in boldface type. An activity value <1.0 indicates inhibition of enzyme activity; a value >1.0 indicates activation of enzyme activity. Data represent the mean of three determinations from one experiment. H19C91, anti-carcinoembryonic antigen antibody. The sequences of gp120 peptide and NS peptide were given in *Materials and Methods*.

35% decrease). Both D101S-I2 and D153G-I2 are activated by the mAbs, with the level of activation ranging from 115% to 185% of the activity in the absence of antibody. Thus, as with the AP hybrid proteins containing the HIV gp120 epitope, the HCV core hybrid in wild-type AP is inhibited upon antibody binding, and HCV core hybrids in the D101S and D153G AP mutants are activated upon antibody binding. Modulation does not appear to be dependent on the epitope inserted and is not specific to a particular mAb–epitope combination. The phenomenon of inhibition and activation is general, although the magnitude of the change in enzyme activity does vary with the mAb–epitope combination.

The level of modulation may be governed in part by antibody affinity or by the fraction of antibody that is functionally active. At higher concentrations of anti-core antibody (230–350 nM), the modulation with most of the antibodies could be increased to 170% and 200% of the activity in the absence of antibody for D101S-I2 and D153G-I2, respectively. To determine if there was a correlation between binding affinity of a hybrid protein–mAb complex and level of modulation of enzyme activity obtained, the binding affinity of D153G-I2 and several anti-HCV core mAbs was determined using a Biacore instrument (Table 3). Anti-core mAb 14-188-104 and mAb 14-726-217 have similar affinity for D153G-I2 (K_d = 0.8–1.0 × 10^{-6} M). The difference in the level of modulation with these mAbs is likely due to differences in the fraction of active protein (Table 3). In contrast, mAb 14-1287-252 has a lower affinity for D153G-I2 (K_d = 5.5 × 10^{-6} M) but a greater fraction of it is active (87%). The difference in the level of modulation for mAbs 14-726-217 and 14-1287-252 is likely due to the difference in binding affinity and not the difference in fraction of active protein.

The specificity of modulation of the HCV core hybrid proteins was determined by using peptide competition and an antibody not specific for the epitope insert. A peptide corresponding to amino acids 57–64 in HCV core (Gln-Pro-Arg-Gly-Arg-Arg-Gln-Pro-Ile-Pro) was used as a competitor of antibody modulation. Fig. 2 shows the results for mAb 14-726-217 with wild-type AP, API2, D101S-I2, and D153G-I2. The peptide has no effect on the enzyme activity of the hybrid proteins in the absence of the anti-core mAb. The addition of peptide to API2, D101S-I2, or D153G-I2 blocks modulation of enzyme activity in the presence of the mAb. Modulation of the HCV hybrid proteins by the other five anti-HCV core mAbs of the hybrid proteins is also blocked by the presence of the peptide (data not shown). In addition, modulation of API2, D101S-I2, or D153G-I2 is specific to antibodies against the inserted epitope; the anti-gp120 mAb has no effect on their enzyme activities (Fig. 2).

Properties of AP-Epitope Hybrid Proteins. Table 4 lists the AP–epitope hybrid proteins that were constructed, the kinetic constants, and temperature half-lives for the proteins. The K_m values are similar for all the proteins. The presence of the point mutations D101S and D153G in wild-type AP has a large effect on k_{cat}; both increase the rate of substrate turnover almost 5-fold. The introduction of these point mutations into API1 results in increased k_{cat} values for D101S-I1 (1.8-fold) and D153G-I1 (1.5-fold) relative to API1, but the k_{cat} values are approximately one-half those of the D101S and D153G mutants. The HCV epitope inserted into wild-type AP (API2) slightly reduces k_{cat}. The introduction of the D101S point mutation into API2 results in a 2.5-fold increase in the k_{cat} of D101S-I2 relative to API2; however, the k_{cat} of D101S-I2 is only one-third that of the D101S mutant. D153G-I2, which contains the D153G point mutation in API2, has a k_{cat} value that is the same as API2 but only one-eighth that of D153G.

The thermal stability of the AP hybrid proteins is an indication of their relative structural stability. The stabilities of

Table 2. Modulation of HCV hybrid protein activity by anti-HCV core antibodies

	Relative enzyme activity					
Anti-HCV mAb	WT	API2	D101S	D101S-I2	D153G	D153G-I2
No mAb	1.0	1.0	1.0	1.0	1.0	1.0
mAb 14-726-217	0.95	**0.83**	1.03	**1.67**	1.07	**1.85**
mAb 14-947-104	0.99	**0.83**	0.97	**1.39**	1.05	**1.60**
mAb 14-153-462	0.99	**0.65**	1.01	**1.52**	**1.18**	**1.73**
mAb 14-1287-252	0.97	0.96	0.99	**1.16**	1.03	**1.21**
mAb 14-188-104	0.96	**0.83**	0.95	**1.30**	1.07	**1.42**
mAb 14-1269-281	0.94	0.87	ND	**1.50**	ND	**1.69**

The hybrid proteins (5 nM) were assayed in the absence and presence of anti-core antibody (35 nM). Enzyme activity is expressed relative to the activity of the hybrid protein in absence of antibody. Enzyme activity values shown in boldface type differ from no antibody controls by more than 15%. An activity value <1.0 indicates inhibition of enzyme activity; a value >1.0 indicates activation of enzyme activity. Data represent the mean of three determinations from one experiment. ND, not determined.

Table 3. Binding affinity of D153G-I2 and anti-core mAbs

Hybrid protein	mAb	K_d, M	% mAb active
D153G-I2*	14-726-217	0.8×10^{-6}	60
D153G-I2†	14-188-104	1.0×10^{-6}	22
D153G-I2‡	14-1287-252	5.5×10^{-6}	87

*Averages of three separate experiments with 3–6 different concentrations of D153G-I2 (0.25–10 μM) used per experiment.
†Averages of two separate experiments with six different concentrations of D153G-I2 (0.5–10 μM) used per experiment.
‡Values from one experiment with seven different concentrations of D153G-I2 (1–20 μM).

wild-type, D101S, and D101S-I1 are similar ($t_{1/2}$ = 69–71°C). The mutations in D153G and API1 both destabilize the proteins, lowering the $t_{1/2}$ values to 53°C and 58°C, respectively. The combination of D153G and API1 to create D153G-I1 further destabilizes the protein structure ($t_{1/2}$ = 39°C). In D153G and D153G-I1, metal-binding affinity is lower, and this may account for the loss in thermal stability.

DISCUSSION

The structure of the active site of AP (11) is shown in Fig. 3. Residues 407 and 408, between which the epitopes are inserted, are highlighted; the distance from the phosphorus atom to the insertion point (N atom of residue 407) is 15.2 Å. If the epitope inserts are assumed to be in an extended-loop conformation, the central residues of the loop would be positioned 20–25 Å from the insertion site and 35–40 Å from the phosphate. (Neither the structures of the loops nor their orientation in space is known; they may extend away from the active site, fold into the cleft surrounding the active site, or lie on the surface of the protein.) For comparison, the distance from the phosphorus atom to the atom furthest away from it on the same subunit of the enzyme (the CA atom of residue 1) is 43 Å and the distance across the binding domain of an antibody (F_v) is roughly 30–40 Å. By considering the distance between the epitope loop and the phosphate-binding site in the active site of the enzyme, it seems likely that the mechanism for modulation involves conformational changes around the active site rather than steric hindrance of substrate. Steric effects due to antibody binding up to 40 Å from the active site should be minimal, if there are any. It is worth noting the similarities between residues 101 and 153; both are aspartic acid residues that are symmetrically positioned at approximately equal distances from the phosphate and the insertion point (between aa 407 and 408). In addition, both residues are involved in forming the hydrogen bond network around the active site (formed by residues 101, 166, 153, and 328).

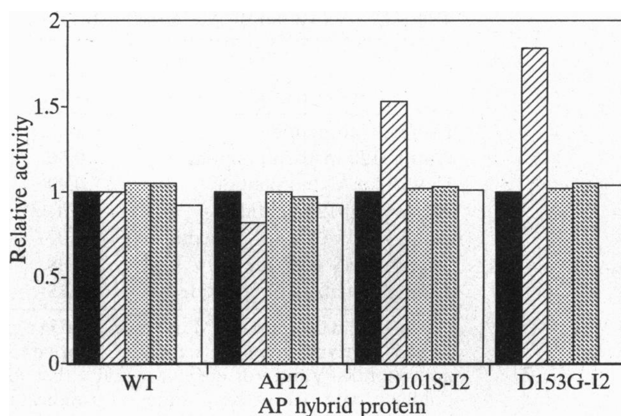

FIG. 2. Modulation of HCV core hybrid proteins is specific. The hybrid proteins (5 nM) were assayed in the absence and presence of antibody (35 nM) and/or peptide (4 μM). Enzyme activity is expressed relative to the activity of the hybrid protein in the absence of antibody and peptide. For each protein: ■, no mAb/no peptide, ▨, mAb 14-726-217/no peptide; ▩, no mAb/HCV peptide; ▧, mAb 14-726-217/HCV peptide; □, anti-gp120 mAb/no peptide. WT, wild-type AP.

In the hybrid proteins, the point mutations D101S and D153G have similar effects when introduced into wild-type AP; the point mutations cause structural changes that increase k_{cat} relative to the hybrid protein with the epitope in wild-type AP. However, the insertion of epitopes has an inhibitory effect on k_{cat} when inserted into the D101S and D153G mutants; k_{cat} values are decreased relative to the protein without the epitope. This is consistent with the active sites of D101S and D153G being less rigid and more sensitive to structural changes around the active site.

The fact that modulation is altered from inhibition to activation by single amino acid changes in the active site of AP supports a mechanism for modulation due to structural alterations in or around the active site upon antibody binding rather than the antibody sterically blocking the active site. One can envision that structural alterations that occur upon antibody binding at the region of the epitope are transmitted through the protein structure to the active site located at least 15 Å away. Minor alterations to the structure of the enzyme can cause dramatic changes in the level of enzyme activity. Antibody binding near a wild-type active site results in changes that reduce enzymatic activity, while binding antibody near the mutant active sites causes alterations that increase activity—e.g., increasing or decreasing the affinity for phosphate is known to affect k_{cat} (3–6). For a given AP–HCV core hybrid protein, several different mAbs have the same effect: either all

Table 4. Properties of AP–epitope hybrid proteins

Protein	AP mutation	Epitope insert*	K_m, μM†	k_{cat}, sec^{-1}†	$t_{1/2}$, °C†
Wild type	—	—	19.9 ± 3.1	6.2 ± 1.1	70
D101S	Asp-101 → Ser	—	17.6 ± 3.0	30.1 ± 1.6	71
D153G	Asp-153 → Gly	—	19.2 ± 1.8	31.2 ± 1.6	53
API1	—	HIV gp120	18.0 ± 1.3‡	9.3 ± 0.2‡	58
D101S-I1	Asp-101 → Ser	HIV gp120	12.9 ± 1.0	16.4 ± 0.6	69
D153G-I1	Asp-153 → Gly	HIV gp120	13.9 ± 1.2	13.7 ± 0.4	39
API2	—	HCV core	18.7 ± 1.0	4.0 ± 0.3	ND
D101S-I2	Asp-101 → Ser	HCV core	18.3 ± 1.0	10.1 ± 1.0	ND
D153G-I2	Asp-153 → Gly	HCV core	9.8 ± 2.5	4.0 ± 0.6	ND

ND, not determined.
*Thirteen-amino acid HIV gp120 epitope sequence, Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr; 15-aa HCV core epitope sequence, Arg-Lys-Thr-Ser-Glu-Arg-Ser-Gln-Pro-Arg-Gly-Arg-Arg-Gln-Pro.
†Values determined as described in ref. 9.
‡Values from ref. 9.

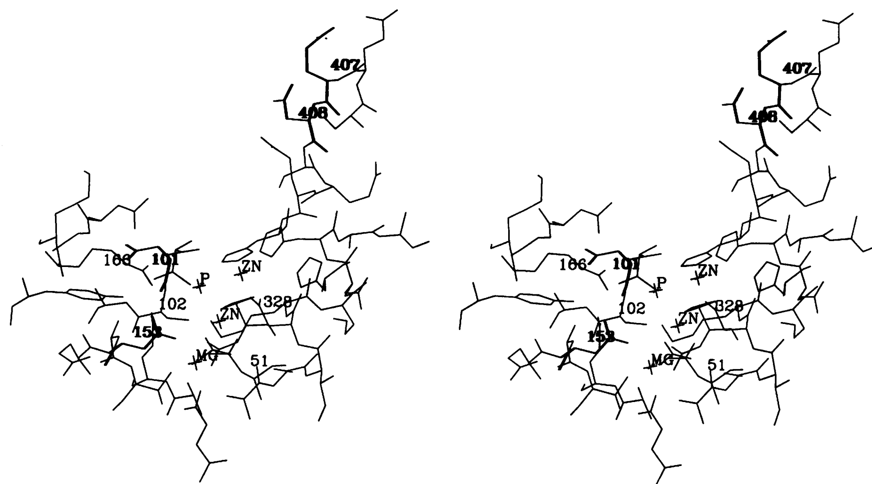

FIG. 3. Structure of the active site of AP [protein data base entry 1ALK (11)]. All amino acids within the 24-Å diameter spherical zone around the active site of subunit A of the enzyme are shown. The highlighted residues were mutated as described in the text; the mutations include either point mutations at positions 101 or 153 or an insertion of 13 or 15 aa between residues 407 and 408. The positions of the phosphate group and metal atoms are indicated by star symbols, together with P for phosphate, ZN for zinc, and MG for magnesium.

inhibit or all activate hybrid enzyme activity. Therefore, modulation is not due to how a particular antibody bound to the epitope affects the active site—i.e., a specific contact between the antibody and AP that causes a specific structural change in or near the active site.

Inhibition or activation of enzymatic activity of AP–epitope hybrid proteins by anti-epitope antibodies is a general phenomenon. Modulation has been demonstrated with two different epitope inserts and several different mAbs. This demonstrates that modulation of the enzyme activity of the AP–epitope hybrid proteins is not specific to a particular epitope sequence or to a particular antibody–epitope combination. While the magnitude of modulation varied for the antibody–epitope complexes, all the antibodies inhibited the enzyme activity of the AP–epitope hybrid protein and activated the D101S–epitope and D153G–epitope hybrid proteins. The magnitude of change in enzyme activity upon antibody binding may be due in part to the binding affinity of the antibody–epitope complex and the fraction of mAb that is functional.

The AP–epitope hybrid proteins are signaling proteins that detect the presence of an antibody by regulating the level of enzyme activity. We have shown that signaling can be in the form of either an increase or a decrease in enzymatic activity upon formation of the bound antibody complex. The consistent trend of activation or inhibition independent of the amino acid sequence of the inserted epitope and independent of the antibody indicates that modulation is a general phenomenon and should work for many epitope–antibody pairs. As such, the described hybrid system might be useful for homogeneous assays for detection of proteins (12). The observed modulation (2- to 3-fold) should be sufficient for a practical application, since current homogeneous assays exhibit this level of modulation—e.g., enzyme-multiplied immunoassay technique (EMIT) and cloned enzyme donor immunoassay (CEDIA) with digoxin. Engineered signaling proteins may be useful for detecting other macromolecules such as receptors, hormones, and proteins, to tag macromolecules *in vivo*—e.g., tagging a receptor on a cell surface (13)—and for allosteric regulation of enzymatic function.

We thank J. Tyner for providing antibodies, K. Jaffe and T. Leung for providing peptides, O. Glenbocki for constructing the D153G mutant, and G. Randall for assistance with protein purification. We thank C. Abad-Zapatero, Mark Hayden, and members of the Protein Engineering Laboratory for helpful discussions and critically reading the manuscript and M. Klass for support.

1. Sowadski, J. M., Handschumacher, M. D., Murthy, H. M. K., Foster, B. A. & Wyckoff, H. W. (1985) *J. Mol. Biol.* **186,** 417–433.
2. Mandecki, W., Shallcross, M. A., Sowadski, J. & Tomazic-Allen, S. (1991) *Protein Eng.* **4,** 801–804.
3. Chen, L., Neidhart, D., Kohlbrenner, W. M., Mandecki, W., Bell, S., Sowadski, J. & Abad-Zapatero, C. (1992) *Protein Eng.* **5,** 605–610.
4. Janeway, C. M. L., Xu, X., Murphy, J. E., Chaidaraglou, A. & Kantrowitz, E. R. (1993) *Biochemistry* **32,** 1601–1609.
5. Matlin, A. R., Kendall, D. A., Carano, K. S., Banzon, J. A., Klecka, S. B. & Solomon, N. M. (1992) *Biochemistry* **31,** 8196–8200.
6. Murphy, J. E., Xu, X. & Kantrowitz, E. R. (1993) *J. Biol. Chem.* **268,** 21497–21500.
7. Brennan, C., Christianson, K., Surowy, T. & Mandecki, W. (1994) *Protein Eng.* **7,** 509–514.
8. Ogata, N., Alter, H. J., Miller, R. H. & Purcell, R. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3392–3396.
9. Chang, C. N., Kuang, W.-J. & Chen, E. Y. (1986) *Gene* **44,** 121–125.
10. Hass, G. M., Bolling, T. J., Kinders, R. J., Henslee, J. G., Mandecki, W., Dorwin, S. A. & Shively, J. E. (1991) *Cancer Res.* **51,** 1876–1882.
11. Kim, E. E. & Wyckoff, H. W. (1991) *J. Mol. Biol.* **218,** 449–464.
12. Khana, P. (1991) in *Principles and Practice of Immunoassay*, eds. Price, C. P. & Newman, D. J. (Stockton, New York), pp. 326–364.
13. Langen, H. T. & Taylor, J. W. (1992) *Proteins Struct. Funct. Genet.* **14,** 1–9.