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Modulation of Structure and Function of Enzymes and Other Biologically Active Proteins by Their Specific Antibodies

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Introduction

In nature, the primary role of antibodies is limited to tagging foreign antigens and thereby initiating their ultimate destruction by other components of the immune system. In some cases, the interacting antibodies have the capacity to neutralize the biological activity associated with the foreign antigens. To ensure efficient tagging and neutralization, the immune system generates antibodies to multitudes of different epitopes on the surface of the antigen – a polyclonal response. Each of these antibodies is specific for a single epitope and is originated in a pre-programmed clone of B-cell lymphocytes which, upon exposure to 'their' antigen and a rather complex chain of events associated with the immune system, produce and secrete their unique antibodies. Once the clonal origin of antibodies was revealed, attempts to clone B cells were initiated as it was recognized that the *in vitro* utilization of antibodies could be greatly increased, since a uniform antibody preparation could be generated from the cloned B cells. White cell cloning procedures were well established at the time, the major obstacle being the inability of B cells to survive, propagate and produce antibodies *in vitro*. This obstacle was finally removed in the late 1970s when Kohler and Milstein (1975) developed a new technology to 'immortalize' B cells. Briefly, a mouse is immunized with the appropriate antigen to elicit a standard polyclonal response. Once a positive polyclonal response is verified, the stimulated and differentiated B cells are harvested from either the mouse spleen or lymph nodes and fused with mouse myeloma cells to create stable hybrid cell lines that can be

Abbreviations: APAAP, alkaline phosphatase–anti-alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; Hex A and Hex B, heat labile and heat stable isoenzymes of *N*-acetyl-hexosaminidase; hGH, human growth hormone; IgG, immunoglobulin G; K_d, dissociation constant; MAb, monoclonal antibody; MW, molecular weight; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PAP, peroxidase–anti-peroxidase; SDS, sodium dodecyl sulphate; t-PA, tissue plasminogen activator.

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propagated *in vitro* (a capacity that is absent in original B cells). This new feature (stable hybrids) enables one to clone and selct individual cell lines originated in a single B cell. As the new hybrid cell lines retain the capacity of the original parent B cell to produce antibodies, the antibodies are monoclonal (i.e. they are specific for a single epitope and can be produced in large quantities and with consistency *in vitro*).

The unique capacity of antibodies to react selectively and with relatively high affinity (an extended exposure to the antigen eventually yields high-affinity antibodies: $K_d < 10^{-9}$ M) with their antigen, has inspired scientists over the last three decades to formulate innovative uses for antibodies not always consistent with their role in nature. Some of the most important include the use of antibodies for the determination of the antigen concentration in a mixture with other proteins (as is the case for hormones in plasma), or for the purification or elimination of the antigen from such a mixture.

Current immunological techniques allow the generation of antibodies to practically any peptide or protein, including autologous protein. Most if not all of the naturally occurring peptides and proteins could be broadly defined as biologically active. The common characteristic of all biologically active proteins is that they possess a specific structure that enables them to interact specifically with other molecules. For enzymes, these specific interactions result in direct catalysis of specific chemical reactions. The interactions of other classes of biologically active proteins and peptides such as hormones, cytokines, etc, result in either stimulation or inhibition of a complex cascade of biological events that involve enzymes.

This chapter will review applications where antibodies are utilized (out of context of the immune system) to form interactions between antibodies and biologically active proteins for the purpose of modulating their activity. It will also address a related phenomenon, enzyme–protein (inhibitor) interactions. But first, the nature of protein–protein interactions will be described using antibody–antigen interaction as a model system.

Antibody–antigen interactions

Enzymes, due to their direct catalytic activity (which can be measured easily), motivated scientists to use them as models to study protein structure–function relations. Hence, some of the best described and studied proteins are enzymes. When advances in X-ray crystallography technology made it possible to study the interaction between antibodies and their antigens, most of the 'first-wave' studies selected an enzyme as the model antigen. Davies, Sheriff and Paldan (1988) recently reviewed X-ray crystallography studies concerning the interaction between hen egg lysozyme and the Fabs of three monoclonal antibodies (MAbs), as well as the interaction of neuraminidase of influenza virus with a Fab of one of its monoclonal antibodies. It was concluded that the interactions are tight, and all water molecules are excluded from the area of contact, which measures around 700 Å^2 (Sheriff *et al.*, 1987). If this is typical of the size of the contact area in antibody–antigen interactions, it will dictate an epitope comprising more than one continuous

oligopeptide (Barlow, Edwards and Thornton, 1986), so that several discontinuous oligopeptides or residues (three for lysozyme) which serve as an epitope would be cross-linked by the antibody reaction. As many as 3 salt links, 10 hydrogen bonds and 74 van der Waals interactions are involved in the case of lysozyme (Sheriff *et al.*, 1987). While the specificity of the antibody will be determined primarily by the individual side-chain reactions, hydrogen bonding and van der Waals interactions, the affinity of the association and the reduction in free energy of the antigen is primarily due to hydrophobic interactions (Rees *et al.*, 1988).

Although these studies employed enzymes as proteins and ignored the consequences of this interaction on enzyme activity and structural integrity, they provide information which allows us to postulate the outcome of this interaction. For example, the extensive cross-linking driven by hydrophobic interactions should result in stabilization of the folded structure of the antigen. Also, it is interesting to note that the thermodynamic parameters determined for the antibody–antigen interaction in these studies are consistent with stabilization of the folded state of the antigen. As the average affinity of monoclonal antibody–antigen binding is around (10^8 M^{-1}), this interaction could lead to a reduction in free energy of the antigen of about 10 kcal mol^{-1} (40 kJ mol^{-1}) (Rees *et al.*, 1988). In a general thermodynamic sense, this is sufficient to confer increased stability, since the difference in free energy between the folded and unfolded states of active globular proteins is in the range 5–15 kcal mol^{-1} (20–60 kJ mol^{-1}) (Tanford, 1970).

Enzyme–protein interactions and their effect on enzyme structural and functional integrity

Antibody–enzyme interactions belong to the larger class of interactions which are described as protein–protein non-covalent associations. In nature, enzymes are involved in protein–protein interactions that in a broad thermodynamic sense resemble antibody–antigen interactions. These interactions can confer stability by excluding water from the interaction area on the enzyme surface, thereby reducing the free energy and driving the reaction towards the folded state (Chothia and Janin, 1975). Certain thermophilic organisms confer stability on their enzymes by elaborating protective macromolecules such as peptides and polyamines (Nakamura *et al.*, 1978; Prasad and Maheshwari, 1978; Oshima, 1982). While multimers or enzyme aggregates are often more stable and active than the constituent monomers (Mozahev and Martinek, 1984). Although protein–enzyme interactions can increase the resistance of the enzyme to thermal unfolding, it could also result in the total inhibition of enzyme activity, as is the case for subtilisin–subtilisin inhibitor complex (Takahashi and Sturtevant, 1981). Subtilisin inhibitor is a protein which binds specifically and stoichoimetrically with subtilisin resulting in the total inhibition of subtilisin. The transition temperature (folded to unfolded state) for subtilisin-inhibitor complex was 20°C higher than for the free enzyme, but this high degree of protection was not beneficial as the enzyme–inhibitor complex was not biologically active. More recently, Swead-

ner (1991) described another stabilizing aspect of the enzyme–inhibitor system, using trypsin and its soybean inhibitors. The interaction of soybean trypsin inhibitors with trypsin and the mechanism that is responsible for the efficient inhibition of trypsin is well documented. The interaction is tight, excluding all water from the interface (Rühlmann *et al.*, 1973; Sweet *et al.*, 1974), with very low dissociation constants (10^{-10}–10^{-14} M). Again, as for subtilisin, this association results in the total inhibition of trypsin enzyme activity, but confers a significant increase in resistance to thermal unfolding (Donovan and Beardslee, 1975). Moreover, Sweadner (1991) showed that the inactive trypsin in this complex was capable of withstanding the irreversible denaturing effect that exposure to sodium dodecyl sulphate (SDS) has on the free trypsin and 'paradoxically' regained its activity in the presence of two very powerful inhibitors. In this case, the extreme structural rigidity imposed by the inhibitor is attenuated by SDS to a certain extent, so that the enzyme regains its activity in an environment in which free enzyme would lose its activity in a matter of seconds.

Antibody–enzyme interactions

There is good reason to believe that the interaction of an enzyme with its specific polyclonal antibodies will, in most cases, result in a significant increase in structural integrity and resistance to thermal unfolding of the enzyme molecules. The effect of this interaction on functional integrity (the activity of the enzyme) is dependent on the location and the role of the epitopes (at which the antibodies bind) in the enzyme activity. It is impossible to predict the extent of the inhibition, or if it will occur at all – every enzyme has to be tested individually.

Most studies in the late 1960s and early 1970s dealing with the effect of specific antibodies on enzyme activity were concerned with enzyme characterization and with differences between wild-type and mutant enzymes, as well as isoenzymes. In probably the earliest study, Burnett and Schmidt (1921) reported that catalase in a complex with anti-catalase (antibodies to catalase) retained full activity, although the complex precipitated. Almost 20 years later, Tria (1939) and Campbell and Fourt (1939), unaware of the report by Burnett and Schmidt, reproduced their results. In the following years, similar studies with other enzymes were published; for example, Cohn and Torriani (1952) made the same observation for β-galactosidase, Stenberger *et al.* (1970) for peroxidase and Cordell *et al.* (1984) for alkaline phosphatase.

For many years, antibody–enzyme complexes have been used very effectively in immunocytochemistry applications. The peroxidase–anti-peroxoidase (PAP) and the alkaline phosphatase–anti-alkaline phosphatase (APAAP) systems have distinct advantages with regard to the reactivity of the antibodies and the enzyme activity when compared with their equivalent covalently conjugated immuno-reagents.

This phenomenon (i.e. that enzymes can retain full enzymatic activity even while in a precipitated complex with their antibodies) was considered an

aberration. General statements such as 'the interaction of enzymes with their specific antibodies generally leads to a reduction in their enzymatic activity' (Ben-Yoseph, Geiger and Arnon, 1975; Solomon *et al.*, 1984) are standard in papers dealing with antibody–enzyme interactions, and they are based on a review by Arnon (1973). The author of the current review believes that in most cases there is only a limited initial loss of activity (10–20%), as described by Melchers and Messers (1970), Ben-Yoseph, Geiger and Arnon (1975), Zyk (1973) and Shami, Rothstein and Ramjeesingh (1989), and that total loss of activity due to an interaction with specific polyclonal antibodies is the exception rather than the rule. The absence of significant inhibition in some (if not most) cases could be explained by a combination of the following: (1) the active site of the enzymes used is a 'blind' spot to the immune system; (2) the inhibitory antibodies are of lower affinity and/or quantity and their binding to the enzyme active site is sterically hindered by adjacent high-affinity antibodies; (3) the low molecular weight synthetic substrates used in the tests are less susceptible to the steric interference imposed by inhibitory antibodies.

However, as soon as the generation of monoclonal antibodies to enzymes became possible in the early 1980s, reports describing total loss of activity by utilizing selected monoclonal antibodies became common. Early reports inclue those by Cotton *et al.* (1980), Frackelton and Rotman (1980), Park *et al.* (1980), Mather *et al.* (1980), Ross, Reis and Joh (1981), Webster, Hinshaw and Laver (1982) and Fambrough, Engel and Rosenberry (1982). Strong indications regarding the powerful utility of monoclonals in enzyme research were found by Solomon *et al.* (1984), who generated a battery of monoclonal antibodies to carboxipeptidase A (an enzyme exhibiting peptidase activity as well as esterase activity). Of the 25 clones stabilized, 2 produced antibodies that selectively inhibited peptidase activity by more than 80%, 2 selectively inhibited the esterase activity by 50%, 10 had no effect or only a slight effect (less than 20%) on both activities, and 11 had a partial effect (20–40% inhibition) on both activities. It is interesting to note that polyclonal antibodies to carboxypeptidase generated in rabbits were capable of only partial inhibition of the enzymatic activity (Amiraian and Plummer, 1971), although in all probability the polyclonal mixture contained industrial antibodies capable of total inhibition when employed alone. Some of the possible reasons for the reduced effectiveness of polyclonal antibodies are listed above.

Mounting evidence has shown that in many cases enzymes in complex with their specific polyclonal antibodies retain their full catalytic activity. This has set the stage for a more comprehensive use of antibodies in enzyme research dealing with the effects on the enzyme activity profile. The activity profile of an enzyme is a composite of activities obtained by gradually changing physical, chemical and biological parameters (such as temperature, pH, substrate, ions, proteolytic enzymes, etc).

Mutated enzymes or isoenzymes may exhibit reduced reaction rate when compared with their respective wild-type enzymes. 'New' calibration of the optimal conditions is generally ineffective in 'reviving' their activity. The

degree of mutation has been monitored by interacting the mutant enzymes with antibodies generated to the wild-type enzymes and comparing it to the interaction of wild-type enzymes with the same antibodies. It was shown that in some cases the mere interaction of a mutant enzyme with antibodies to wild-type enzyme was sufficient to restore a large proportion of the lost enzyme activity, as reported by Rotman and Celada (1968) and Messer and Melchers (1969). They attributed this effect to the enforcement of proper (wild-type) folding by the interacting antibodies. Heat-inactivated acetylcholinesterase was partially reactivated following the addition of its specific antibodies (Michaeli *et al.*, 1969); again, the same general mechanism was implicated.

The next, logical, step taken in these early studies was to determine the effect of pre-treatment (of wild-type enzymes, mutant enzymes and isoenzymes with their respective specific polyclonal antibodies) on the capacity to resist increasingly 'denaturing' conditions such as heat, pH and proteolytic enzymes. These studies revealed that:

1. Mutant catalases sensitive to heat and mild alkalinity were stabilized by interacting them with antibodies generated against the wild-type catalase (Feinstein *et al.*, 1971).
2. Wild-type β-galactosidase and 10 of its 11 mutants, which were complexed with the antibodies generated against the wild-type enzyme, exhibited increased resistance to heat inactivation. The greatest shift (12°C) and overall highest value (66.5°C) of the transition temperature was recorded for the wild-type enzyme (Melchers and Messer, 1970).
3. The heat labile and heat stable isoenzymes of *N*-acetyl-hexosaminidase (Hex A and Hex B, respectively) were both stabilized against heat inactivation by their respective antibodies (Ben-Yoseph, Geiger and Arnon, 1975).

As these early studies were not concerned with the stabilization of enzymes for practical purposes, this short-lived and restricted burst of activity in the field of antibody–enzyme interaction dissipated without follow-up. Even the emergence of monoclonal antibodies in the late 1970s and early 1980s did not stimulate new activity in this field.

In the mid and late 1980s, advances in X-ray crystallography and protein engineering provided new tools for studying the structure–function relations of proteins, and antibody–antigen interactions became a popular model system, with most models utilizing enzymes as model antigens. The ability to modify almost at will the primary structure of antigens and antibodies by standard recombinant DNA techniques, coupled with the ability to achieve high-resolution images of protein tertiary structure by X-ray crystallography and, more recently, by nuclear magnetic resonance (NMR), has revived interest in this field.

In 1987, we embarked on the development of practical applications utilizing the unique properties of antibody–antigen interactions to confer resistance to inactivation and stabilize commercially valuable proteinaceous antigens. To test our hypothesis, we used a number of model enzymes and treated them with their specific antibodies (polyclonal antibodies for

α-amylase, glucoamylase and subtilisin, and selected protective, non-inhibitory, monoclonal antibodies for L-asparaginase, singly or in mixtures) and subjected them to physical, chemical and biological inactivation. The results presented here are drawn from Shami, Rothstein and Ramjeesingh (1988, 1989), Shami *et al.* (1991) and Ramjeesingh *et al.* (1992).

Briefly, enzymes in their pure form were pre-incubated overnight at 4°C, with increasing concentrations of either their pure monoclonal antibodies or with the IgG fraction for their polyclonal antibodies, the control enzyme samples were 'spiked' with equivalent amounts of non-immune human IgG and pre-incubated under the same conditions. both enzyme samples were then exposed for varying lengths of time to various inactivation procedures.

Enzyme functional integrity can be irreversibly disrupted by physical, chemical and biological influences. We selected heating, freezing and lyophilization as physical methods, oxidation and the effects of ethanol as chemical methods, and proteolytic degradation by trypsin as a biological method. Protection was assessed by determining the residual activity of the enzyme, at the end of the exposure period. The optimal antibody–enzyme concentration ratio for protection was then determined for each model enzyme–AB system. This ratio was then used for all further protection experiments.

THERMAL INACTIVATION

This parameter was assayed with the enzymes α-amylase (*Figure 1A, B*), glucoamylase and subtilisin. In all three cases, the retained activity of the antibody complexed enzymes was substantially higher than that of the free enzyme. We recorded increases in short-term (5 min) heat resistance (i.e. the temperature for 50% inhibition) of 6.0°C for subtilisin, 16.0°C for glucoamylase and 20.5°C for α-amylase (*Table 1*). Long-term heat resistance was defined in terms of the half-life at a selected temperature. The temperature was selected so as to maximize the protective effect for each enzyme. Generally at this temperature, the short-term heat resistance for the protected enzyme was still close to 100% of the control, whereas for the unprotected enzyme it was well below 50%. The selected temperatures for long-term thermal inactivation ranged between 65°C and 70°C. As much as a 240-fold increase in the half-life of α-amylase was noted, and 60-fold and 65-fold increases were recorded for glucoamylase and subtilisin, respectively (*Table 1*). While the dramatic increase in the long-term heat resistance of α-amylase was associated with an impressive shift of 20.5°C in short-term heat resistance, a 16.0°C shift for glucoamylase resulted in less of an increase in long-term heat resistance than that recorded for subtilisin with only a 6.0°C shift.

Economically speaking, long-term heat resistance is the more important feature for industrial enzymes. However, for practical reasons, in most reports 'the most promising stabilized enzyme preparations' are designated as such, based on screening for short-term temperature-dependent heat resistance. Our results show clearly that care should be taken to analyse all preparations for time-dependent heat resistance as well, since larger tempera-

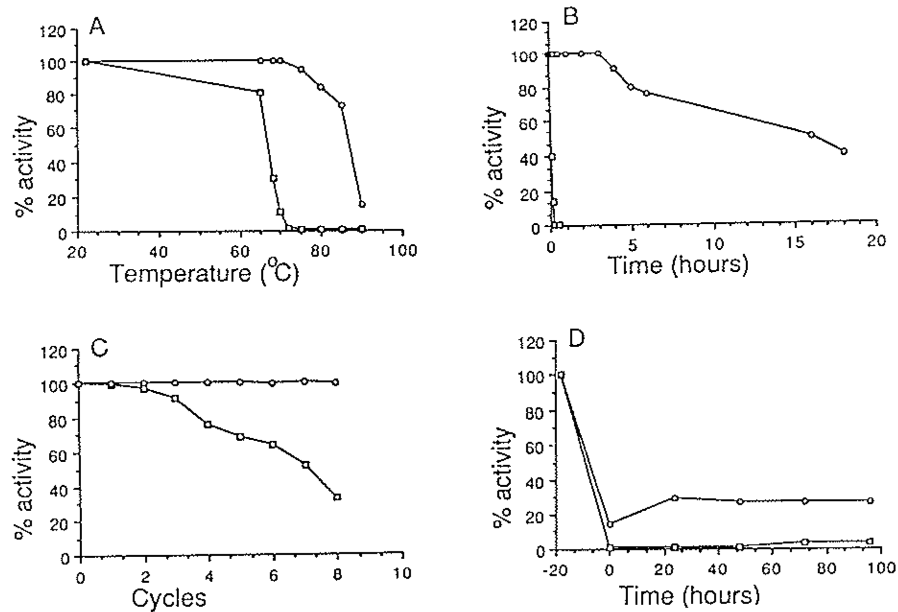

Figure 1. Protection of α-amylase by specific antibodies against physical disruption. From Shami *et al.* (1991).
(A) Heat inactivation of α-amylase. The results of residual activity (100% activity = activity at 23°C) of protected and unprotected α-amylase samples as a function of temperature. The same symbols for protected and unprotected enzymes are used throughout.
(B) Time-dependent inactivation of α-amylase at 70°C. The results of residual activity of protected and unprotected enzyme samples as a function of incubation time at 70°C.
(C) Protection of α-amylase against inactivation by freezing and thawing. The residual enzyme activities (expressed as percent of pre-treatment enzyme activity) for protected and unprotected samples as a function of number of freezing and thawing cycles.
(D) Lyophilization effect on protected and unprotected α-amylase. The results are expressed as percent of pre-treatment enzyme activity.

ture shifts are not always associated with a proportionally longer half-life.

FREEZING AND THAWING

After eight cycles of freezing and thawing, there was no significant loss of activity for antibody-protected α-amylase, whereas the unprotected enzyme lost over 65% of its initial activity (*Figure 1C*). This protective capacity might provide greater flexibility in exploiting industrial enzymes, providing an additional option for storage.

Table 1. Thermostability of enzyme–antibody complexes[a]

Enzyme	T(°C)	Half-life			T(°C) for 50% inhibition		
		A Free enzyme	B Enz–AB complex	B/A	C Free enzyme	D Enz–AB complex	D–C T(°C) shift
α-amylase[b]	70	4 min	16.0h	240	66.5	87.0	20.5
Glucoamylase[c]	66	3 min	3.0h	60	57.5	73.5	16.0
Subtilisin[c]	65	4 min	4.3h	65	63.0	69.0	6.0

[a] From Shami *et al.* (1991).
[b] Data obtained from *Figure 1A, B*.
[c] Data for glucoamylase and subtilisin were obtained using experimental protocols similar to those described for α-amylase.

LYOPHILIZATION

The unprotected α-amylase lost almost all of its enzyme activity after lyophilization, while the antibody-protected enzyme retained approximately 15% of its initial activity (*Figure 1D*). Twenty-four hours after reconstitution, the protected sample had recovered 25% of its initial activity, an activity that was maintained for a period of 96 h. The unprotected α-amylase did not exhibit significant recovery of activity. Of all the physical disruption forces tested, the protection provided by antibodies against lyophilization was the least impressive. However, as we tested only one enzyme for this effect, it is possible that other enzymes may benefit more from such protection, again allowing an additional storage option.

OXIDATION

The short-term (5 min) exposure of subtilisin to increasing concentrations of NaOCl revealed that the antibody protection factor was largest (81 *vs* 60%) at 0.05% (w/v) NaOCl. However, upon longer-term exposure (30 min) of subtilisin to 0.05% (w/v) NaOCl, only 25% of its original activity was retained, while the antibody-protected enzyme retained close to 80% of its activity (*Figure 2*). It would appear that following the initial damage due to oxidation, the protected enzyme remained unchanged at around 80%, whereas the unprotected enzyme kept on losing its activity. Oxidation is a major inactivation force in nature. *In vitro*, enzymes are rather vulnerable to oxidation even under normal atmospheric conditions, let alone if extra oxidant is added to the system, as is the case with the detergent enzyme subtilisin, which has to function in the presence of the bleach-oxidant, NaOCl. The action of subtilisin could be enhanced greatly by an increase in resistance to both high temperature and oxidation. Antibodies are capable of providing this dual protection.

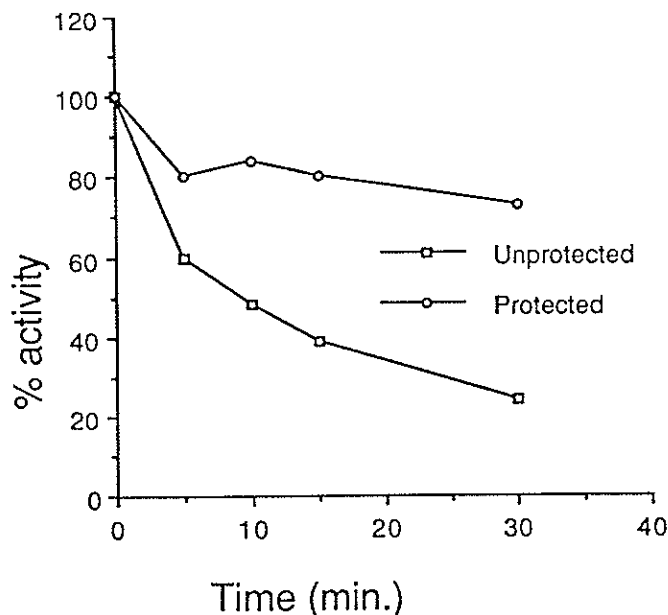

Figure 2. Time-dependent effect of oxidant (sodium hypochlorite: NaOCl). The residual enzyme activity, expressed as percent of the rate obtained for the corresponding untreated enzyme preparations, is plotted as a function of incubation time for the protected and unprotected enzyme. From Shami *et al.* (1991).

THE EFFECTS OF ETHANOL

Glucoamylase pre-incubated with 2.5% ethanol for 25 h retained only 10% of its original activity, whereas the antibody-protected enzyme retained 98% of its activity (*Figure 3*). The enzymatic conversion of sugars to ethanol is a well-known process. As the alcohol concentration rises in the system, some of the enzymes involved are subject to product inhibition by ethanol. In the brewing of beer, this phenomenon is actually exploited to terminate the reaction so that the level of ethanol does not rise above the required level. However, the production of industrial alcohol is geared towards the maximization of ethanol levels, so resistance to ethanol could be beneficial.

PROTECTION WITH ANTIBODIES AGAINST PROTEOLYTIC INACTIVATION

The clinical application of protein-based pharmaceuticals is rapidly increasing. For many, however, the prolonged maintenance of appropriate blood levels is a problem because of their relatively short *in vivo* half-life. Since the most common form of *in vivo* inactivation for many proteins is enzymatic proteolytic degradation (Tombs, 1985), any method of reducing proteolytic susceptibility might lead to reduced dosage, improved clinical efficacy and management, and reduced costs.

To a certain degree, proteolytic inactivation is related to inactivation by unfolding. In 'folded' proteins, the cleavages are limited in number because

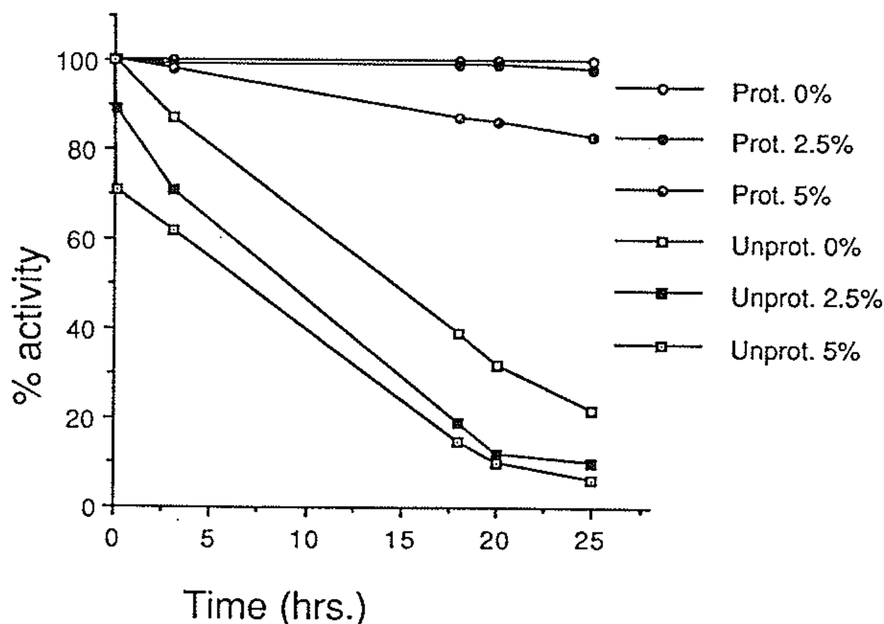

Figure 3. Time-dependent effect of ethanol on glucoamylase enzyme activity. The absorbance of the untreated protected (Prot.) and the untreated unprotected (Unprot.) samples at time zero were taken as 100% activity. The results for the time-dependent effect at 37°C of 0%, 2.5% and 5% ethanol on protected and unprotected glucoamylase are shown. From Shami (1991).

few or no proteolytic sites are exposed and such cleavages do not necessarily result in inactivation (Stoops *et al.*, 1978), due to the fact that catalytic domains are highly organized and more stable, whereas the peptide loops that link the domains are relatively unstructured and, therefore, more susceptible to digestion. In unfolded proteins, on the other hand, all potential cleavage sites are exposed, so that extensive degradation and loss of biological activity occurs (Mclendon and Radany, 1978). As to be expected from such considerations, a significant correlation between thermal stability and resistance to proteolytic inactivation has been noted. Thermostable enzymes from thermophilic bacteria are, for example, more resistant to proteolysis than are similar enzymes from mesophilic organisms (Daniel *et al.*, 1982). Enzymes with higher melting temperatures (T_m) are more resistant than those with a lower T_m (Mclendon and Radany, 1978; Daniel *et al.*, 1982). It can be inferred, therefore, that any process that confers thermal stability – as is the case with antibodies – might also confer some degree of protection against proteolytic inactivation.

One approach to stability which has achieved some success involves covalent conjugation of other macromolecules, such as polyethylene glycol (Abuchowski and Davis, 1981) or albumin (Poznansky *et al.*, 1982), to target proteins. The limitations, however, are that random covalent coupling in many cases results in reduced activity and that the overall stabilization is considerably lower than that achieved with the aid of specific antibodies.

The first use of antibodies to protect biologically active protein

(L-asparaginase) against biological inactivation (proteolysis) was documented by Zyk (1973). In the case of L-asparaginase, polyclonal antibodies partially inhibited the enzyme, but the residual activity was more resistant to proteolysis (Zyk, 1973). More recently, a MAB proved capable of blocking the single trypsin cleavage site of alkaline phosphatase (Jemmerson and Stigbrand, 1984), but because this cleavage is non-inhibitory (R. Jemmerson, pers. comm.), protection against inactivation was not demonstrated.

Monoclonal antibodies can protect L-asparaginase against inactivation by trypsin

Ramjeesingh *et al.* (1992) reported on the capacity of selected MAbs to provide protection against trypsin inactivation of the anti-leukaemic agent, L-asparaginase (Broom, 1961), an enzyme that converts L-asparagine to L-aspartic acid. Of 20 clones (IgG class) producing antibodies against L-asparaginase, 6 were selected for further study based on their production of MAbs with relatively high binding affinities for the enzyme (estimated by ELISA). Their protective capacity was assessed by comparing the rate of conversion of the substrate (L-asparagine) by enzyme complexed with each MAb, challenged with trypsin, to the rate of the control (enzyme spiked with equivalent amounts of non-immune human IgG, challenged with trypsin). The substrate consumption curves are shown in *Figure 4*. Neither non-immune human IgG (used as a control) nor any of the MAbs were inhibitory in the absence of trypsin. Trypsin treatment of the free enzyme reduced the substrate conversion rate to a minimal level (6% of the control), equivalent to 94% inactivation. Little or no protection against trypsin inactivation was afforded by the non-specific IgG or by three of the MAbs (Nos 19, 33 and 35). On the other hand, one MAb (No 12) was highly effective, preserving 72% of the control rate, and two others (Nos 29 and 34) provided intermediate protection (33 and 20%, respectively). In the experiment, substrate was depleted within 40 min (MAb No 12). During two additional cycles of substrate addition and depletion, MAb No 12 preserved the 72% level of activity, indicating that protection had been maintained for at least 120 min, despite the continuous presence of trypsin. The most effective MAb (No 12) was selected for further study. Its subclass type was determined as IgG2b with κ light chain.

TRYPSIN AND MAb CONCENTRATION

The protective action of MAb No 12 was influenced by the trypsin concentration and by the ratio of MAb to enzyme. Protection is defined as either the difference between, or the ratio of, the activity of MAb protected enzyme and the unprotected enzyme when challenged with trypsin. The highest protection (around 72% and a protection ratio of 12) was obtained with 37.5 units of trypsin per ml (*Figure 5*), using a ratio of MAb to enzyme tetramer of 10 : 1 (*Figure 6*). Increasing the ratio to 20 : 1 raised the overall activity of the protected sample, but provided no additional protection (as defined above)

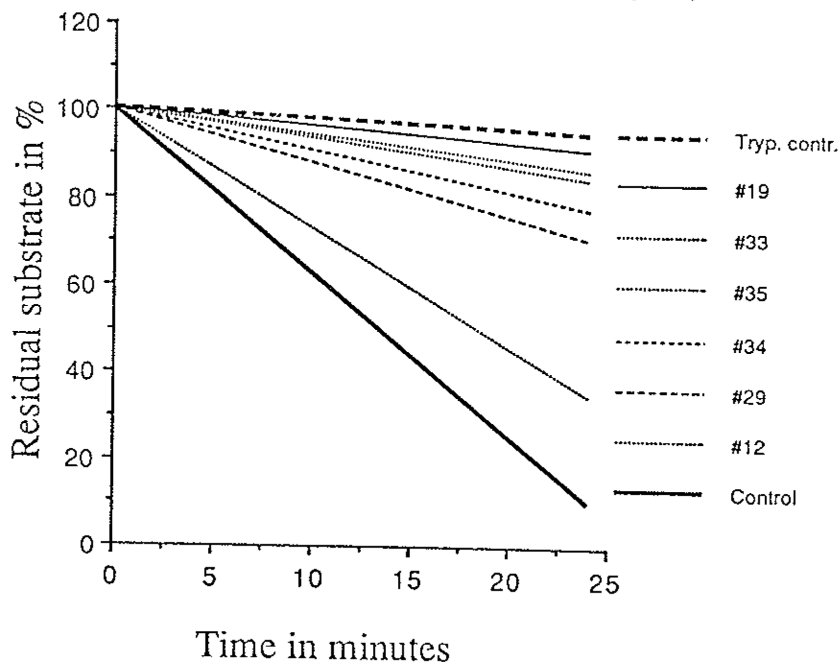

Figure 4. Screening for protective monoclonal antibodies. L-Asparaginase was protected with MAbs (Nos 19, 33, 35, 34, 29 and 12) and challenged with trypsin. Tryp. contr., free L-asparaginase spiked with equivalent amounts of non-immune human IgG and challenged with trypsin; Control, free L-asparaginase. Enzyme activity was determined as described in the experimental protocol. Residual substrate curves were generated by continuously monitoring the preparations at 197 nm (three readings per min per sample) and using the apparent initial rates obtained for residual substrate concentrations above 10% (100 μM) that corresponds in this case to 10 times the K_m value. From Ranjeesingh *et al.* (1992).

due to a similar increase in activity in the control sample (resulting from non-specific protection from high protein concentration).

STOICHIOMETRY OF THE MAb-ENZYME COMPLEX

Protection of enzyme activity implies that a MAb-enzyme complex has been formed. Direct evidence for complex formation was obtained by use of goat anti-mouse antibodies. Over 90% of asparaginase activity was precipitated, indicating that most of the enzyme tetramers were complexed with at least one MAb (MIgG). At the optimal 2.5 : 1 molar ratio of MAb: asparaginase monomer (*Figure 5*), 95% occupancy of binding sites would be predicted from the 2×10^{-8} M dissociation constant, K_d (determined by immunoprecipitation). Maximal protection, however, was only about 72%, which leads us to speculate that one monomer per tetramer was unprotected due to steric hindrance.

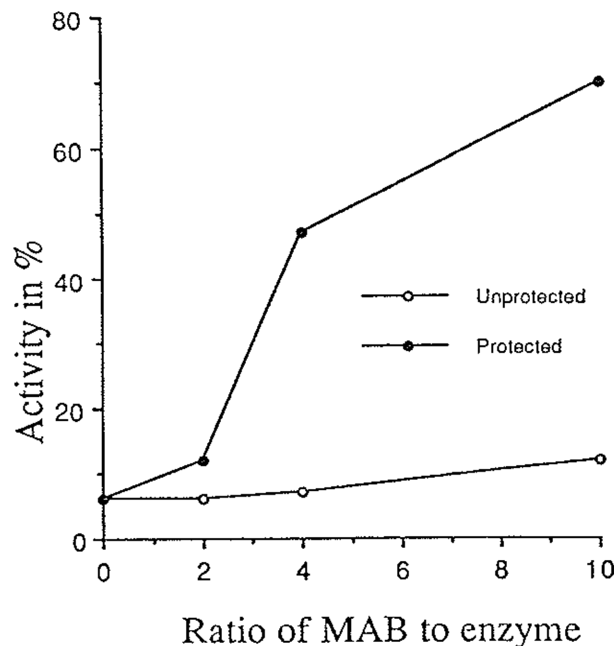

Figure 5. The protective effect of MAb No 12 as a function of trypsin concentration. Protected, L-asparaginase protected with MAb No 12; unprotected, L-asparaginase spiked with non-immune human IgG. Both preparations were challenged with increasing amounts of trypsin. Activity is expressed as percent of activity of the corresponding zero trypsin samples. From Ramjeesingh *et al.* (1992).

Mode of trypsin inactivation and location of its cleavage site

Based on SDS polyacrylamide gel electrophoresis, trypsin reduced the apparent molecular mass of asparaginase monomers by 3–4 kDa to about 31 kDa (*Figure 7*). On the other hand, enzyme protected by MAb No 12 was resistant to this cleavage. According to Maita, Morokuma and Matsuda (1974), *Escherichia coli* L-asparaginase II contains 321 amino acid residues per monomer (MW 34 080) in a sequence that was deduced from sequencing 27 non-overlapping peptides produced by trypsinization, and whose arrangement was deduced from cyanogen bromide peptides. A more recent sequence (Jenning and Beacham, 1990) based on molecular analysis of the isolated gene suggests 326 residues per monomer (MW 34 549).

To locate the inhibitory cleavage site, the major tryptic product (about 31 kDa) was recovered from the gel column and subjected to analysis. Its N-terminal sequence was Val-Gly-Val-Glu-Asn-Leu-. Only a single matching location was found, according to both published sequences (Maita, Morokuma and Matsuda, 1974; Jenning and Beacham, 1990) and the tryptic map. As expected, it is adjacent to lysine 29, since trypsin cleavages result in lysine or arginine C-terminal peptides. Thus the tryptic peptide must be cleaved from the N-terminal region and has a calculated molecular mass of 2647 Da rather than the 3–4 kDa estimated from the difference in mobility in SDS-gel (an inaccurate procedure) noted above. The role of the N-terminal domain of

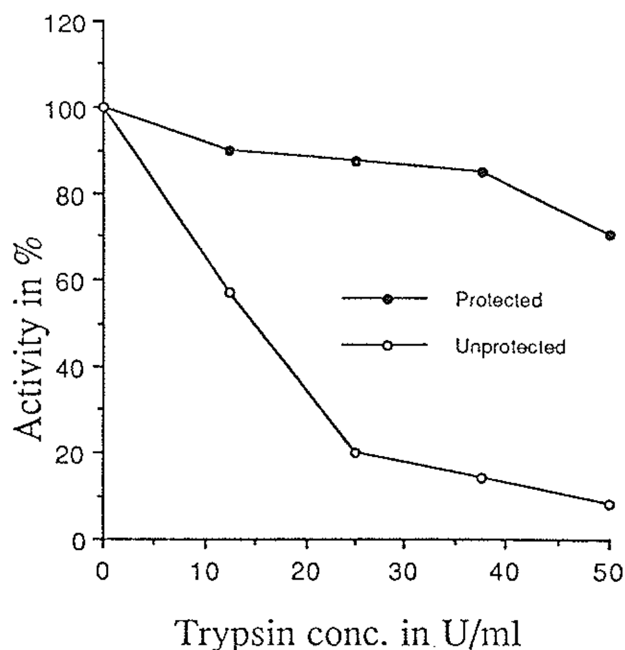

Figure 6. Protective effect of MAb no 12 against trypsin as a function of its concentration ratio with the enzyme. Protected and unprotected preparations were prepared, challenged with 37.5 U ml^{-1} trypsin and assayed for activity. Protected: L-asparaginase protected with increasing amounts of MAb No 12; the ratio of MAb to enzyme is expressed as a molar ratio of MAb to tetramer enzyme. Unprotected: L-asparaginase spiked with equivalent amounts of human IgG. Activity is expressed as percent of rate of substrate consumption for the free enzyme, unchallenged with trypsin (shown in Fig. 4). From Ranjeesingh *et al.* (1992).

L-asparaginase, has been revealed by a recent study that determined its crystal structure (Swain *et al.*, 1993). This study proposed that this tetrameric enzyme is a dimer of identical intimate of dimers and the active sites, are located between residues of primarily the N-terminal and to a lesser extent residues of the C-terminal domains of monomers belonging to an intimate dimer. Thus, cleavage of the 2647 Da N-terminal peptide leads to deletion of some of the residues comprising the active site, resulting in inactivation of the enzyme and MAb No 12 protects activity by preventing the cleavage (*Figure* 7).

MAbs Nos 12 and 29, which both afford some protection, appear to bind to non-overlapping epitopes. MAb No 12 binds equally well to the intact enzyme monomer and the 31.9 kDa trypsin product (*Figure* 5). MAb No 29, on the other hand, binds to the intact enzyme monomer but not to the 31.9 kDa trypsin product. This could be interpreted as meaning that its epitope is largely located on the cleaved 2647 Da fragment. However, it is also possible that its epitope is actually on the 31.9 kDa fragment and the cleavage of 2647 Da by trypsin has disrupted the epitope so that it is no longer recognizable by the MAb.

This study demonstrates that a non-inhibitory monoclonal antibody can be selected which provides substantial and sustained protection against proteo-

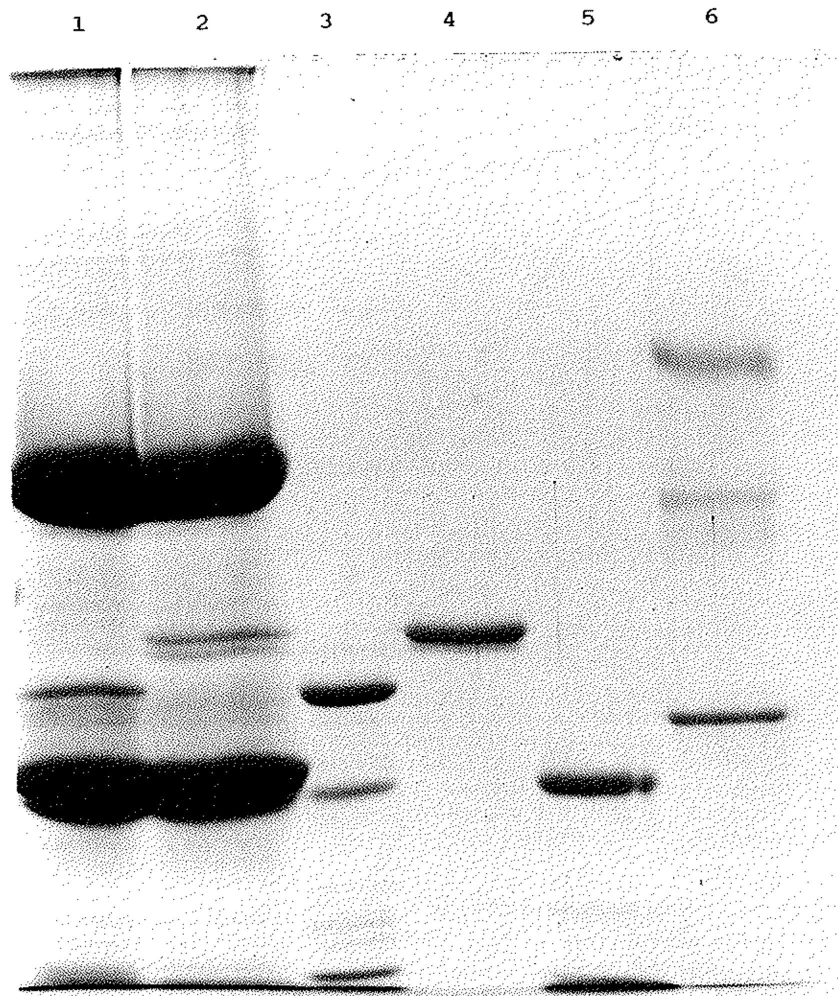

Figure 7. Comparison of protected and unprotected L-asparaginase by SDS-PAGE (10%). Effect of trypsin. Lane 1, L-asparaginase (5 μg) spiked with 10 : 1 molar ratio of non-immune human IgG, plus trypsin. Lane 2, L-asparaginase (5 μg) protected with 10 : 1 molar ration of anti-asparaginase MAB No 12, plus trypsin. Lane 3, free L-asparaginase (10 μg) treated with trypsin. Lane 4, free L-asparaginase (10 μg). Lane 5, trypsin (10 μg). Lane 6, molecular weight standards: BSA 66 kDa, ovalbumin 45 kDa, carbonic anhydrase 29 kDa. Trypsin-treated samples for lanes 1–3 were prepared as follows: 10 μg of L-asparaginase and 113 μg of non-immune human IgG (lane 1) or anti-asparaginase MAB No 12 (lane 2) were combined in 25 mM NaCl, 10 mM Tris pH 7.8 (final volume 100 μl; lane 3 sample just in buffer) and incubated over night at 4°C. The following day, samples for lanes 1–3 were treated with trypsin (20 μl, 20 μg, 200 U) for 5 min at 37°C, followed by addition of 20 μl of 'laemmli sample solubilizing solution', boiled for 5 min and concentrated by evaporation under nitrogen to a final volume of 40 μl, 20 μl (per lane) of which was used for SDS-PAGE (10%) according to Laemmli (1970). From Ramjeesingh *et al.* (1992).

lytic inactivation of L-asparaginase by trypsin. Of the six MAbs that were tested, none was inhibitory to the enzyme reaction. The most simple explanation is that none bind in the region of the active site of the enzyme in a fashion that blocks the access of the small size substrate (L-asparagine) to the

active site. Polyclonal antibodies, which were reported by Zyk (1973) to partially inhibit, might hinder substrate interactions by binding to several epitopes near the active site.

Maximal protection was about 72% with a protection ratio of 12. On the other hand, at the optimal 2.5 : 1 ratio of MAb : asparaginase monomer, 95% occupancy of binding sites would be predicted from the 2×10^{-8} M dissociation constant (K_d). This discrepancy leads us to speculate that full occupancy (4 MAbs per tetramer) may be prevented by steric hindrance.

Other MAbs with similr binding affinity do not afford protection, indicating that binding *per se* is not sufficient. Presumably, in order to afford protection, a MAb must bind to a particular region of the protein's surface. However, more than one epitope may be associated with protection. For example, the most protective MAb (No 12) binds to the 31.9 kDa trypsin product, but No 29, which provides more modest protection, does not. At the most, only a small overlapping fraction of each MAb's binding area (estimated to be 26 × 19 Å: Sheriff *et al.*, 1987) can be involved in the protection action, or perhaps each MAb protects independently with no overlapping epitopes.

In vivo use of complexes comprising antibodies and biologically active proteins

Immunoassay is the most common application that utilizes monoclonal antibodies to form complexes with active proteins. While practically all the current 'approved' immunoassay applications are for *in vitro* diagnostics, there have been a number of studies concerning as yet unapproved *in vivo* diagnostic uses. However, since the purpose of this interaction (in immunoassay) is not the modulation of the biological activity of the target protein, this application will not be addressed here.

The ultimate utility of hormones, cytokines and clinically important enzymes is *in vivo*, in therapeutic applications. As mentioned earlier, their short half-life in circulation is a major drawback which hinders effective utilization. It is evident that, as proteins, their stability and activity can be modulated by complexing them with either specific polyclonal or monoclonal antibodies. The employment of these complexes *in vivo* has been studied by several groups.

Due to its antiviral, antiproliferative and immunomodulatory properties, interferon-αA was one of the first cytokines to be produced by recombinant DNA techniques. Pharmacokinetic studies conducted by Rosenblum *et al.* (1985) revealed that non-inhibitory anti-interferon monoclonal antibodies, when complexed with interferon prior to intravenous administration to rats, increased the half-life of interferon-αA three-fold (from 60 to 188 min). In this case, the monoclonal antibody was selected based on its capacity to interact with interferon, without neutralizing its activity. It is likely that reduced urinary excretion is due to the increased size of the complex (× 150 000) and is the main reason for the increase in half-life.

In another instance, 'Potentiation of the somatogenic and lactogenic

activity of human growth hormone (hGH) monoclonal antibodies' was reported by Aston *et al.* (1986). They attributed this potentiation effect to the possibility that hGH in complex with a certain antibody may be capable of binding only to a particular class of hGH receptors, thereby enhancing the biological activity associated with these receptors. They carried out the experiments *in vivo*, both for the somatogenic assay (in Snell dwarf mice) and the lactogenic assay (Pigeon crop sac bioassay). They reported that only direct injection into the crop sac resulted in enhancement; injections at other locations were ineffective. This led them to conclude that systemic mechanisms such as prolonged half-life are not involved in the enhancement phenomenon.

Animal models for the therapeutic use of enzyme–antibody complexes have been used to test the effectiveness of both polyclonal and monoclonal antibodies. Surprisingly, Hadely, Lindmann and Sato (1987) found that L-gulunolactone oxidase (an enzyme that catalyses the final step in ascorbic acid – vitamin C biosynthesis), administered by the intraperitoneal route as a complex with either homologous or heterologous polyclonal antibodies to guinea pigs, doubled their survival time when maintained on an ascorbic acid deficient diet. The use of a polyclonal enzyme–antibody complex had no adverse reaction (with regard to allergic or toxic response), although in this study the enzyme–antibody complex was further fortified by covalently cross-linking it with gluteraldehyde. Other enzymes, including serum cholinesterase, L-asparaginase and histidase, were also tested in this study.

A more recent study by Dunn (1991) utilized a t-PA (tissue plasminogen activator)–anti-t-PA monoclonal antibody covalently linked complex to extend the half-life of t-PA in rabbits. However, as this study only monitored the presence of t-PA by ELISA (rather than t-PA enzymic activity), the reported extension in half-life may be irrelevant, as inactivated t-PA molecules are recognized as well by ELISA.

General features

Substantial protection of enzymes and other biologically active proteins by specific antibodies has been demonstrated against physical inactivation (high temperatures, freezing and thawing, and lyophilization), chemical inactivation (low, pH, oxidation with NaOCl and ethanol and biological inactivation (proteolysis). Considering the number of proteins that have been successfully protected by either polyclonal or monoclonal antibodies, it would appear likely that, in principle, protection can also be afforded to most other proteins. However, practical considerations restrict the use of antibodies to mainly non-industrial *in vitro* applications, such as stabilization of research and diagnostic reagents and stabilization of growth factors used in either research or large-scale production involving cell culture.

Barriers to the use of antibodies in industrial applications are mainly economic in nature. For example, industrial enzymes are inexpensive reagents and protecting them with antibodies that are four orders of magnitude more expensive is not practical, even if one can increase the half-life by two to

three orders of magnitude. To exploit this protective phenomenon exhibited by antibodies, one would have to integrate via recombinant DNA techniques the antibody features that are responsible for the protection effect of the enzyme molecule, and to create a new modified enzyme that can be produced at the same cost as the native enzyme. This might be accomplished by using existing methodologies. For example, protective non-inhibitory monoclonal antibodies could be converted into single chain antibodies (see review by Wetzel, 1988). In this form, the DNA sequence encoding for the antibody (variable regions) could be fused to the DNA encoding its antigen (the enzyme to be protected) for the construction of a fusion gene, similar to the one described by Chaudhary *et al.* (1989). This fusion gene, upon expression in *Escherichia coli* for example, might produce a chimeric protein comprised of two domains – the first an enzyme and the second a protective single chain antibody – that might provide stabilization by interacting with a vulnerable site on the enzyme.

In clinical applications, there is no economic barrier, as protein drugs are quite often more expensive to produce than antibodies. In clinical applications, it is not the utilization of murine monoclonal antibodies *per se* that is the problem (there are approved MAb drugs on the market), but the fact that in each protected preparation there are three different protein species (i.e. MAb-protein complex, free protein and free MAb), which is a major tumbling block in the process of drug approval. Again to overcome this barrier, the protective features of the antibody would have to integrate into the protein-drug via recombinant DNA, so that new more resistant drugs can be produced.

In principle, non-inhibitory MAbs should be selectable, which would provide protection against proteolysis of any protein-drug, potentially improving its efficacy. The protective MAb could be 'humanized' (Reichmann *et al.*, 1988), converted to a single chain antibody (Bird *et al.*, 1988) and its gene fused with that of the target protein. This would allow the production of a fusion protein containing, for example, a cytokine and a single chain antibody (Chaudhary *et al.*, 1989), connected via an appropriate spacer. The product might be biologically active, proteolysis-resistant, with low immunogenicity, economically producible by a single organism and suitable for clinical use.

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