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Strategy for obtaining non-native protein structures using antibody cross-reactions

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Antibodies made to short peptides or to unfolded forms of proteins are often found to cross-react with intact proteins. These cross-reactions can be used to populate non-native protein conformations, possibly including protein folding intermediates, and the structures of the non-native conformations can be characterized using amide proton exchange and two-dimensional NMR.

1. Introduction

Most single-domain proteins show cooperative unfolding transitions at equilibrium: the cooperativity of protein folding makes it difficult to populate non-native protein structures [1,2]. Nevertheless, protein folding intermediates are known to exist [1,2] and alternative protein conformations have been demonstrated by numerous methods [2–4].

A long-standing problem is how to populate non-native protein conformations (including protein folding intermediates) at high levels, so that their *structures* can be studied. This information is necessary to understand the mechanisms by which proteins fold, and to elucidate how signal transduction through proteins occurs. This paper considers trapping of non-native protein conformations using antibody cross-reactions, and describes an approach for characterizing the trapped species.

2. Antibody cross-reactions

In 1945, Landsteiner [5] reported that antibodies can distinguish native proteins from their denatured counterparts. Since then, the conformational specificity of antibodies has been demonstrated using different proteins in both folded and unfolded forms [6]. Nevertheless, the specificity of antibody-binding reactions is not absolute: when an antibody made to an unfolded protein binds to the native form of the protein or vice versa, it is called a *cross-reaction*.

The classic studies of antibody cross-reactions were carried out by Anfinsen and co-workers [7,8] who studied staphylococcal nuclease (SNase) and its fragments. These experiments were designed to detect extremely low levels of cross-reaction: the DNase activity of SNase was used to assay for SNase that was not inhibited by antibodies.

Although protein fragments of SNase do not show detectable folded structure in aqueous solution [9], the protein fragments can bind antibodies made to native SNase. The affinity for fragments, however, is 10^3–10^4-fold lower than that for intact protein [7]. The low level of cross-reaction was interpreted [7] to be a consequence of the small fraction of fragment molecules in a native-like conformation ($= K_{conf}$). This type of cross-reaction, in which antibodies raised to the *native* form of a protein are used, will not be discussed further in this paper.

Conversely, antibodies to an unfolded fragment of SNase can bind to intact SNase, under condi-

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tions where *isolated* SNase is folded [8]. Binding of the anti-fragment antibodies to intact SNase, however, is 3000-fold worse than that to the fragment. Furie et al. [8] interpreted these results to mean that 1 part in 3000 of intact SNase is unfolded; the equilibrium between native and unfolded states was pulled by the antibodies and the protein unfolded as a result.

In an analogous study using different fragments of myoglobin as immunogens, the extent of cross-reaction (with intact myoglobin) was found to vary significantly [10]. In two of three cases, cross-reaction was better than expected if total unfolding of myoglobin was required, suggesting that in some cases only partial unfolding is needed for cross-reaction to occur.

More recently, it was discovered that antibodies to peptides as short as seven residues could cross-react with intact proteins (for a review, see ref. 11). Nevertheless, most short peptides (< 20 residues) are disordered in aqueous solutions [12]. Lerner and co-workers [13] refer to the cross-reaction of anti-peptide antibodies with intact proteins as the 'order-disorder paradox'.

Can the model of Anfinsen and co-workers explain the order-disorder paradox? Unfortunately, the new generation of immunological methods (e.g., ELISA, Western blotting), used widely to demonstrate anti-peptide antibody cross-reactions, does not measure true affinities. This makes it hard to infer the mechanism of cross-reaction involved.

3. Site-specific perturbants of protein structure

Whereas antibodies against native proteins often recognize 'discontinuous' epitopes, anti-peptide antibodies or antibodies made to unfolded proteins usually have 'linear' epitopes [14]. Thus, overlapping synthetic peptides can be used to map the site-specific linear epitopes (usually 5–7 residues long) that are being considered there.

What criteria can be used to determine if a cross-reacting antibody is a good candidate for use as a site-specific perturbant? *Solution-phase* affinities should be measured. Consider an antibody made to an unfolded protein (anti-U). We can compare the affinity for binding the unfolded protein (U) to the affinity for binding the native protein (N). The following classes of results can be considered:

(i) Anti-U binds N with lower affinity than it binds U, and the difference in affinity is predicted by the free energy for unfolding N [8]. This case is consistent with a model in which complete unfolding of N is required in order for it to bind to anti-U (fig. 1C).

(ii) Anti-U binds N with lower affinity than it binds U, but the difference in affinity is less than predicted by the free energy for unfolding N. This result is consistent with our goal of perturbing the structure of N without completely unfolding it (fig. 1B). The difference in affinities is a measure of the free energy required for the perturbation.

(iii) Anti-U binds N with approximately equal affinity as it binds U. This result might suggest that there is little conformational change in N when it cross-reacts with anti-U (fig. 1A). The epitope might correspond to a region of N that is normally unfolded or very flexible. Alternatively, the epitope may correspond to a region in U that folds readily.

(iv) Anti-U binds N with greater affinity than it binds U. This result would be consistent with one of the models considered by Lerner and co-workers for explaining the cross-reaction of anti-peptide antibodies with intact proteins [15]. In this model, anti-U recognizes the folded form of the epitope, even though an unfolded protein or peptide was used for immunization. The affinity of anti-U for U is less than that for N because the antibody only recognizes the fraction of U that is folded in aqueous solution.

The models discussed above are only *interpretations* of the thermodynamic results: there are many other factors that can affect these affinities. Nevertheless, knowledge of the thermodynamics of a given cross-reaction is useful before characterizing it by structural methods. The kinetics of association also provides information about the mechanism of antibody cross-reactions (see, e.g., refs. 7, 16 and 17).

Our results (to be published) with polyclonal antibodies against unfolded bovine pancreatic trypsin inhibitor (U-BPTI, made by reduction of

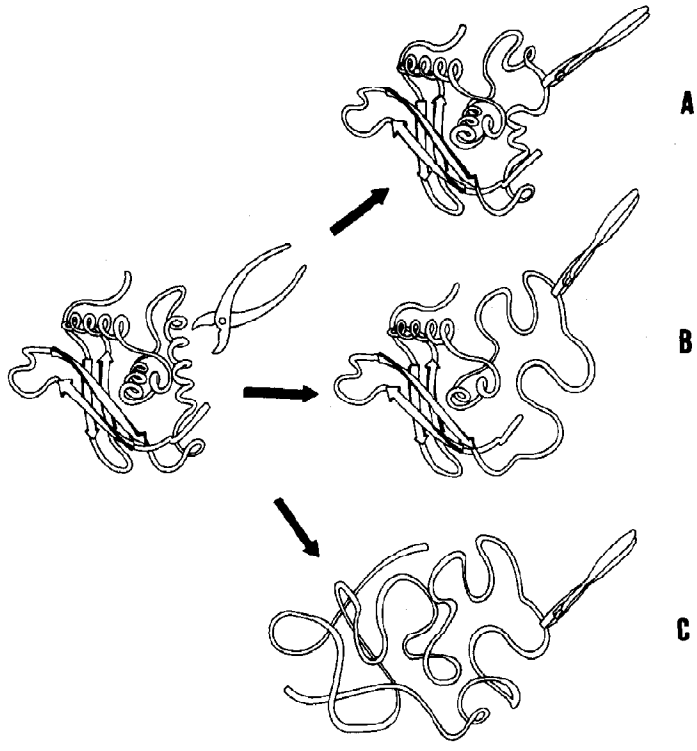

Fig. 1. Schematic drawing of different antibody cross-reactions: (A) little or no change in protein structure; (B) perturbation of protein structure without complete unfolding; (C) complete unfolding of the native protein.

the three disulfide bonds and blocking of thiols with iodoacetamide) indicate that there are several cross-reacting epitopes. Affinity measurements suggest that these cross-reacting epitopes fall into class ii above: the affinity for N is less than that for U, but not as small as predicted by the free energy of unfolding for BPTI. Encouraged by these results, we are now making monoclonal antibodies to U-BPTI.

4. Characterization of altered protein conformations using amide proton exchange and NMR

How does one characterize the structure of a protein while it is bound to a cross-reacting antibody? Ultimately, one would like to obtain a high-resolution crystal structure of the complex between the protein and the binding domain of the antibody (F_{ab}). It may prove very difficult, however, to obtain co-crystals, especially if significant portions of the protein are disordered when bound to the antibody. Moreover, one wants a simpler method to characterize cross-reacting antibodies at the initial stages.

One way to obtain relatively high-resolution information about the conformation of the protein while bound to a cross-reacting antibody is to carry out amide proton exchange and then to assay the extent of exchange at individual amide protons using two-dimensional NMR. The NMR assay is carried out after dissociation of the protein from the antibody: direct NMR studies of antibody-protein complexes are not practical. This method gives information about many sites in the protein and does not require reassignment of the NMR spectrum for the protein.

Amide proton exchange has been used extensively to study protein dynamics [18,19] and protein folding [20–22]. In the case of BPTI, there are 25–30 protons that are stable enough to permit characterization by two-dimensional NMR [18]. These amide protons exchange at least 10^4-fold faster in the unfolded state than in the folded state.

The degree of protection from exchange for a given amide proton, $[\theta]_P$, has been defined as the ratio of the intrinsic exchange rate, k_{int} (i.e., in the absence of structure), to the observed exchange rate, k_{obs} [21,22]:

$$[\theta]_P = k_{int}/k_{obs}$$

An unfolded amide proton will have $[\theta]_P = 1$, as compared to $[\theta]_P \geq 10^4$ in the native state (for BPTI). Even if only part of the protein unfolds when it binds to a cross-reacting antibody, it should be easy to detect the unfolding event. Changes in *stability* of an amide proton will be reflected by intermediate values of $[\theta]_P$.

The protocol for using amide proton exchange to study antibody cross-reactions is: (i) deuterate the amide protons of the protein using established methods (e.g., see ref. 18); (ii) form the antibody-protein complex in ^2H_2O; (iii) initiate exchange by adding H_2O; and (iv) quench exchange by decreasing the pH (amide proton exchange is acid- and base-catalyzed with a minimum exchange rate near pH 3; see ref. 24).

Fortunately, the pH used to quench amide proton exchange (pH 2–3) is also the pH often used to dissociate antigen-antibody complexes [25]. Thus, it is possible to quench exchange and dissociate the protein-antibody complex in the same step; dissociation does not result in loss of label from amide protons. The protein is separated rapidly from the antibody (or F_{ab}) at the quench pH using gel filtration.

The extent of amide proton exchange in the separated protein is quantitated by measuring the NH-C$^\alpha$H cross-peak intensities in a COSY spectrum (e.g., see ref. 18). Measurements of cross-peak intensities as a function of labeling time gives exchange rates for individual amide protons in the antibody-protein complex.

5. Concluding remarks

Cross-reacting antibodies can be used as site-specific perturbants of native protein structures. The non-native protein structures can be characterized at equilibrium using different methods; amide proton exchange and two-dimensional NMR can give relatively detailed information. The characterization depends on a comparison of the degree of protection from amide proton exchange, $[\theta]_P$, when the protein is bound to the antibody, as compared to when the protein is free in solution. The primary limitation of the method is that solvent exclusion by the antibody combining site could retard exchange. For example, binding of BPTI by trypsin results in reduced exchange rates for amide protons in BPTI, including some at the trypsin/BPTI interface [26]. The interpretation of results with antibody cross-reactions, therefore, should rely more on amide protons with *increased* exchange rates in the presence of the antibody. An increased rate of exchange is most likely a reflection of structure destabilization or unfolding.

Amide proton exchange can also be used without NMR, using radioactive water ($[^3H]H_2O$) as a labeling reagent [24]. Thus, it should be possible to monitor conformational changes that occur in antibody cross-reactions with proteins that do not have an assigned NMR spectrum.

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