Hydrogen Exchange Analysis of Ligand-Induced Conformational Changes in Fab†

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ABSTRACT: Effects of ligand binding on the conformation of Fab′ have been assessed by measuring the hydrogen exchange behavior of Fab′ derived from sheep or rabbit antibodies. Antibodies involved in calcium-dependent reactions with $(Glu,Ala,Tyr)_n$ were used. In agreement with previous studies, 16–19 apparent combining site associated amide hydrogens do not exchange out from liganded Fab′ while for unliganded Fab′ they have a $t_{1/2}$ of 3.2 h at pH 6. The numbers of these affected hydrogens were independent of pH of exchange in or out or of pH crossover but decreased significantly (10–12 H) for fully reduced and alkylated Fab′ which was of full binding activity. From experimental protocols where effects of combining-site hydrogens were maintained constant throughout exchange out, a group of 6–7 apparently non-combining-site hydrogens was detected whose exchange was retarded upon ligand binding. Analysis of these hydrogens suggests that they represent a group which could be between 17 and 47 in number and be involved in either a 2 → 3 or a 1 → 6 h shift for unliganded and liganded Fab′, respectively. From other experimental protocols, it was found that as many as 12 other non-combining-site-associated amide hydrogens do not exchange in liganded Fab′. These hydrogens have a $t_{1/2} \sim 8$ h at pH 6.0, and about half of them are disulfide sensitive. In addition, some portions of these hydrogens are pH sensitive. This suggests that there is a region of Fab′ away from the combining site whose conformation is sensitive to both ligand binding and pH. The pH sensitivity may be due to Fab′–Fab′ interactions in liganded Fab′. Other structural amide hydrogens ($t_{1/2} > 5$ h) of Fab′ were also found to experience slowed exchange upon ligand reaction. Taken together, these results show that ligand reaction results in a tightening of some parts of the Fab′ structure, not only at the combining site but also in other parts of the molecule. The sum total of these effects encompasses a small percentage of Fab′ structure. These results indicate that 5% of Fab′ amide hydrogens have their breathing reactions altered by ligand reaction, discounting effects at the combining site. Some of the changes observed involve highly structured regions of Fab′ while others are probably in looser parts of the molecule. These findings, which clearly indicate that a ligand induces conformational effects away from the combining site, would be consistent with an allosteric model for antibody although a more complicated model seems appropriate on the basis of other considerations.

An intriguing problem in protein chemistry concerns the effects of ligand binding on protein conformation in regions of the molecule where binding takes place, at regions neighboring the binding site, and at regions considerably distant from the site of binding. Such effects are presumed to be informational as they are purported to transmit signals which result in regulation at the molecular level. Although theoretical models and supportive phenomena which are totally consistent with this concept have existed for many years (Monod et al., 1965), direct experimental measurements of such changes, particularly those distal to the site of ligand attachment, have been difficult to obtain. Currently, our best understanding of ligand-induced conformational changes in macromolecules exists for the oxygenation of hemoglobin. This understanding is based upon two factors: (1) a detailed knowledge of hemoglobin structure provided from X-ray analysis and (2) the experimental feasibility of performing X-ray analysis on liganded hemoglobin (Heidner et al., 1976; Ladner et al., 1977). With this information, the details of hemoglobin ligand-induced conformational change have been analyzed (Baldwin & Chothia, 1979; Moffat et al., 1979). These factors have also been instrumental in the successful formulation of experiments done by other less informative physiochemical techniques and in analyzing them. The elegant hydrogen exchange studies of Englander and co-workers (Englander & Mauel, 1972; Englander & Rolfe, 1973) on the conformation of hemoglobin, for example, were initiated from observations these authors made on molecular models of hemoglobin. In the absence of appropriate molecular models, the assignments of effects to specific regions of a molecule become a difficult task.

Because immunoglobulins are involved in a variety of well-studied sequellae (effector functions), there has been much interest in the conformation of antibody molecules and particularly effects initiated by antigen binding. Extensive studies on a number of antibody systems have led various investigators to consider several possibilities regarding ligand-induced effects (Metzger, 1978). These are (1) that antigen induces conformational changes distal to the combining site (allosteric model), (2) that the topological arrangement of antigenic determinants results in distortion of the antibody molecule, leading to conformational changes away from the combining sites (distortive model), and (3) that antibody binding to antigen results in the juxtaposition of Fc's (Fc aggregation) which is conducive to the binding of other molecules involved in effector functions (associative model). Presently, there is no compelling physical evidence for possibility (1) or (2), and at this time it seems that molecular control mechanisms of the immune system may not involve antibody conformational change, but, instead, control may operate through modulated sequential reactions. Thus, the equilibrium constant of an effector-related (effector-triggering) macromolecule interacting

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with antibody Fc might be enhanced by antigen aggregation of antibody Fc, i.e., through multivalent enhancement of the association constant.

Of the various antigen–antibody reactions which have been examined in studies of ligand-induced antibody conformational change, calcium-dependent reactions are uniquely suited because such reactions involve defined regions on certain macromolecules which require calcium for determinant formation (Liberti et al., 1971). As such, (1) highly purified antibody (or enzymatically derived antibody fragments) can be obtained by elution of charged adsorbants with calcium-chelating buffers, (2) in the absence of calcium nonspecific perturbations of either reaction component on the other can be measured, (3) association or dissociation parameters of antigen–antibody reaction can be measured since reaction is totally controlled, and (4) antibody interaction with multivalent antigen can be examined. The existence of calcium-dependent reactions in populations of antibody directed to random-sequence polypeptides, such as $(Glu^{60}Ala^{30}Tyr^{10})_n$ (Glu-Ala-Tyr),[1] $(Glu^{90}Tyr^{10})_n$, and $(Glu^{60}Ala^{40})_n$, is well-known and has been extensively characterized (Liberti et al., 1971; Liberti, 1975; Liberti & Vickerman, 1977). Calcium-dependent antigen–antibody reactions also exist in populations of antibodies to serum albumins (Frankel & Liberti, 1980).

Some years ago, ligand-induced changes in antibody conformation were examined by performing hydrogen exchange measurements on antibodies to calcium-dependent determinants of Glu-Ala-Tyr (anti-GAT–Ca^{2+}). Hydrogen exchange measurements were done because they are extremely well suited for studying the dynamics of protein conformational change, and, in principle, the status of all amide hydrogens can be measured and analyzed. Also, measurements can be confined to regions of either high or low conformational mobility, and, very importantly, a single component in a multicomponent system can be examined. Data compiled with hydrogen exchange demonstrated that 18–24 amide hydrogens of Fab′ do not exchange upon ligand binding and that 14 of these hydrogens have an approximate half-time of exchange of 3 h at pH 6.0, with the remaining hydrogens exchanging faster (Liberti et al., 1972a). These results were interpreted as the ligand inducing a conformational change at the combining site by freezing segmental breathing of combining site associated amide hydrogens. In accordance with the relatively long half-time of these hydrogens (considering that they are in contact with solvent), it was suggested that combining sites are highly hydrogen bonded and considerably rigid. These Fab′ results, which have since been confirmed by X-ray crystallographic analysis, provided the first indication of the number of antibody residues forming the combining site (Poljak et al., 1973; Segal et al., 1974). From studies done on the effects of ligand binding in soluble antigen–antibody complexes formed in antigen excess, it was also found that antibody amide hydrogens are released in both IgG and $(Fab')_2$ when complexes are formed at moderate levels of antigen excess (3 times and lower) (Liberti et al., 1972b). Thus, it was suggested that a distorted model of antibody conformational change was appropriate, even though these changes apparently take place near the hinge region rather than in Fc.

Recently, we decided to reinvestigate the hydrogen exchange of liganded Fab′ because in previous exchange studies less than half the amide hydrogens of Fab′ were monitored and because we felt that changes in Fc conformation postulated in either the allosteric or the distortive models for antibody should very likely be accompanied by conformational changes in Fab′ away from the combining site. On the basis of these experiments, it will be shown that in addition to the previously demonstrated ligand-induced changes at the combining site ligand binding by Fab′ results in conformational changes away from the combining site. These changes are consistent with the notion that breathing reactions away from the combining site are slowed. In addition, our results suggest that there is a region of Fab′ whose conformation is pH sensitive.

Materials and Methods

Glu-Ala-Tyr (lot M 18G) was obtained from Pilot Chemical Co., Boston, MA. Before use, it was treated by procedures previously described (Liberti, 1975). Glu-Ala-Tyr used in hydrogen exchange experiments was a high molecular weight monodispersed fraction isolated by ion-exchange column chromatography (Liberti & Vickerman, 1978). The preparation of Sepharose immunoadsorbants has also been described (Liberti, 1975).

Antibodies and Fab. Sheep antibodies to Glu-Ala-Tyr were raised by methods previously described (Maurer et al., 1970). Rabbit antibodies were obtained from rabbits selectively bred for a calcium-dependent response. For these experiments, antibodies were obtained from rabbits who were the third generation calcium-dependent responders. Sheep or rabbit antibodies involved in the calcium-dependent reaction were isolated from charged immunoadsorbants by elution with EDTA as previously described. Each was readsorbed and reeluted a second time to ensure purity of preparations. The preparation of Fab′ was achieved via pepsin (37 °C, 14 h) digestion of whole antibodies and subsequent reduction with dithiothreitol (DTT). $(Fab')_2$ was first dialyzed against 0.15 M Tris, 0.15 M NaCl, and 0.002 M EDTA, pH 8.0, N_2 was bubbled through the sample, and then it was reduced with DTT (1.1 mM for native Fab′, 5 mM for reduced Fab) for 90 min at 37 °C. For quantitation of the extent of reduction and for the purpose of monitoring Fab′ concentration in hydrogen exchange experiments, alkylation was done with [1-^{14}C]iodoacetamide (New England Nuclear, Boston, MA) for 60 min at 25 °C in the dark. After these procedures were completed, Fab′ preparations were exhaustively dialyzed against either 0.05 M cacodylic acid and 0.15 M NaCl, pH 6.0 (Cac–saline buffer), or 0.05 M Tris and 0.15 M NaCl, pH 7.8 (Tris–saline buffer), pervaporated to concentrations of 5–8 mg/mL, redialyzed, and stored in aliquots at −70 °C.

Hydrogen Exchange. Hydrogen exchange procedures have been reported in detail (Liberti et al., 1972a,b; Liberti, 1975). Briefly, equilibrium mixtures were prepared by mixing 100–150-μL aliquots of protein solutions with microliter quantities of tritium water (1 Ci/g, New England Nuclear, Boston, MA) to achieve equilibrium activities of 10–100 mCi/mL. Quantities of tritium in equilibrium mixtures were quantitated by triplicate analysis. After appropriate incubation, equilibrium mixtures were made dense by sucrose addition (180 mg/mL) and layered onto appropriately temperature- and buffer-equilibrated calibrated Bio-Gel P-2 columns. Column dimensions (15 × 200 mm) were chosen so that eluted protein was well separated from unexchanged-in tritium water. This enabled determinations of zero time exchange-in values. All

[1] Abbreviations used: Glu-Ala-Tyr, $(Glu^{60}Ala^{30}Tyr^{10})_n$; Glu-Tyr, $(Glu^{90}Tyr^{10})_n$; Glu-Ala, $(Glu^{60}Ala^{40})_n$; anti-GAT–Ca^{2+}, antibodies to calcium-dependent determinants of Glu-Ala-Tyr; EDTA, disodium salt of ethylenediaminetetraacetic acid; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; Tris buffer, 0.05 M tris(hydroxymethyl)aminomethane and 0.15 M NaCl, pH 7.8; Ca^{2+}–Tris, 0.01 M calcium Tris; EDTA–Tris, 0.01 M EDTA Tris; Cac buffer, 0.05 M cacodylic acid and 0.15 M NaCl, pH 6.0; Ca^{2+}–Cac, 0.01 M calcium Cac; EDTA–Cac, 0.01 M EDTA Cac. The immunoglobulin nomenclature is in accordance with *Bull. W.H.O.* (1964).

column operations were times to ensure uniform handling of samples. Exchange-out measurements at later times were determined by using a rapid-dialysis apparatus. All determinations were done in duplicate (some in triplicate) and at 5 °C. All experiments reported were repeated at least once and generally twice. The number of unexchanged hydrogens per molecule was calculated from the Englander equation modified for use with ^{14}C-labeled protein as a means of measuring protein concentration and applicable in a dual-label scintillation counting situation (Liberti et al., 1972a). Data reduction was done on a Wang programmable calculator (program available on request).

Experimental Considerations and Experimental Design

Since Glu-Ala-Tyr at neutral pH has considerably extended structure, and low helix content (Liberti et al., 1971) due to its high negative charge, it would be expected to have negligible amounts of intramolecular hydrogen bonding. Consequently, nearly all its amide hydrogens should exchange as free amide hydrogens and exchange in or out rapidly. This expectation is borne out by experiments done at pH 6.0 and 7.8. When Glu-Ala-Tyr was exchanged in under conditions routinely used for exchanging in antibody or Fab′ and exchange out was started by gel filtration chromatography by the procedure given under Materials and Methods, it was found that Glu-Ala-Tyr emerging from the column was completely exchanged out (2.5–3.5 min). Thus, if Glu-Ala-Tyr was included in Fab′ exchange experiments, i.e., in the equilibrium mixture, it was found to be without effect on the exchange of Fab′. This was also the case when Fab′ was derived from anti-GAT–Ca^{2+}, provided that the initial gel filtration was done in the absence of a antigen–Fab′ interaction, i.e., no calcium, or in EDTA excess. Since this procedure makes the presence of Glu-Ala-Tyr "transparent" with respect to exchange, considerable experimental latitude is possible. For example, exchange-out experiments can be done on Fab′ which had been exchanged in, either in a reacted or in a unreacted condition.

Figure 1 depicts the possible experimental designs which can be employed as consequences of the calcium dependency of this reaction and of the rapid exchange out of Glu-Ala-Tyr considered above. In the figure, Fab′ is depicted, in a simplistic fashion, as having three kinds of amide hydrogens, combining-site amide hydrogens (18–24, depending upon the experimental protocol employed), indifferent amide hydrogens, and possible ligand-affected hydrogens away from the combining site. Species I of the figure designates Fab′ exchanged in in the presence of Glu-Ala-Tyr but in the absence of calcium, i.e., unreacted. This is entirely comparable to exchange-in protocols where Glu-Ala-Tyr is absent. As indicated in the figure, both combining-site and indifferent amide hydrogens exchange in. The area corresponding to non-combining-site ligand-affected hydrogens is left as a question mark. For this exchanged-in species, exchange out can be done with or without antigen–antibody reaction (species II or III, respectively), in which case combining-site amide hydrogens will exchange out with effectively zero rate from species II; i.e., they do not exchange, or they will exchange out with their unperturbed rate from species III. The possible role of ligand-affected hydrogens which by previous experimental protocols appeared to be absent will be examined here. Also shown in Figure 1 is the status of Fab′ exchanged in under conditions of reaction, i.e., with Glu-Ala-Tyr in 10 mM calcium. Since combining-site amide hydrogens do not exchange out with ligand reaction, accordingly, they should not exchange in with reaction (species IV). Those amide hydrogens designated "?" exchange in either perturbed or unperturbed, depending on effects ligand reaction may have on them. As indicated in the figure, species IV can be exchanged out with either Fab′ reacted (species V) or unreacted (species VI). We will show that with these experimental designs information about non-combining-site hydrogens can be obtained.

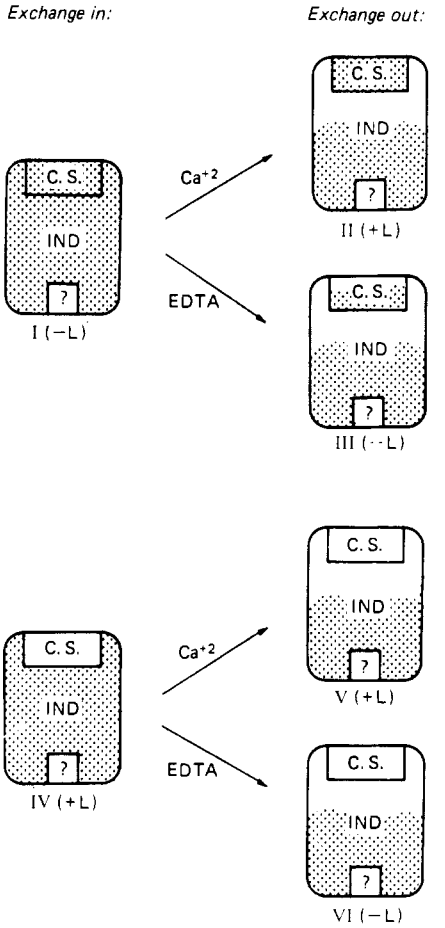

FIGURE 1: Amide hydrogen models and experimental protocols for the exchange of calcium-dependent Fab′. With antigen present, anti-GAT–Ca^{2+} Fab′ can be exchanged in or exchanged out with or without antigen–antibody reaction taking place, +L or −L, respectively. Amide hydrogens of Fab′ are represented by the area of a rectangle subdivided in three fractions corresponding to (1) amide hydrogens of the combining site (C.S.) which are ligand sensitive, (2) indifferent amide hydrogens which are insensitive to ligand interaction (IND), and (3) a group of amide hydrogens away from the combining site designated "?" which may be sensitive to ligand perturbation. For exchange-in species (I and IV), shaded areas correspond to amide hydrogens which exchange in during a period of 19 h. For exchange-out species (II, III, V, and VI), completely shaded areas represent nonexchanging hydrogens while partially shaded areas correspond to amide hydrogens which are exchanging out. Experiments based on these models are shown in Figures 3–6.

Results

Figure 2 shows exchange-out curves of sheep Fab′ anti-GAT–Ca^{2+} in the presence of Glu-Ala-Tyr at 20 times antigen excess at pH 7.8 and in the presence of calcium (reaction) and no calcium (no reaction). The curves diverge apart to a constant difference of about 18 hydrogens per molecule. For the experiment shown, Fab′ exchange in was done in the absence of Glu-Ala-Tyr and at pH 7.8. Nearly identical results were obtained for preparations of rabbit Fab′ anti-GAT–Ca^{2+}. Qualitatively, similar results were obtained for both species of Fab′ when exchanged in and out were done at pH 6.0. Experiments done with Glu-Ala-Tyr present in the exchange-in mixture were without effect on the results of Figure 2.

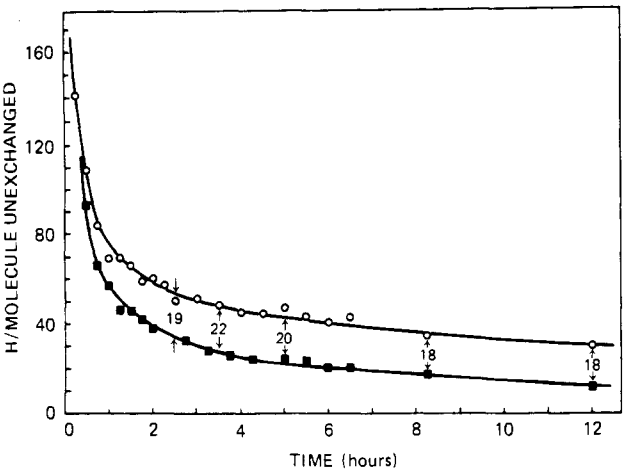

FIGURE 2: Exchange-out kinetics of anti-GAT–Ca^{2+} Fab′ in the presence of 20 M excess Glu-Ala-Tyr at pH 7.8. Fab′ was exchanged in at 4 °C for 19 h in Tris buffer, pH 7.8. Exchange out was initiated on a Bio-Gel P-2 column equilibrated in Ca^{2+}–Cac, pH 6.0. Antigen was added after elution from the column, and dialysis was started against Ca^{2+}–Tris, pH 7.8 (○), or EDTA–Tris, pH 7.8 (■).

For the most part, these results are identical with those from previous studies on other preparations of Fab′. They show clearly that ligand binding prevents the exchange of Fab′ amide hydrogens (combining site associated residues). In contrast to previous results, when measurements in the 2–5-h period (see Figure 2) were done with utmost care, differences in exchange curves of reacting and nonreacting Fab′ appear to go through a maximum before becoming parallel. This suggests that other ligand effects might be operative. To determine if that is the case, it is desirable to minimize the effects of combining-site hydrogens on exchange curves, since in the experiments of Figure 2 these hydrogens are exchanging in one case (bottom curve) and are essentially nonexchanging in the other (top curve). For accomplishment of this, a sample of sheep Fab′ (anti-GAT–Ca^{2+}) at pH 6 was mixed with a 20-fold excess of antigen, tritiated, and immediately aliquoted into one vessel containing an amount of dried calcium chloride required for 10 mM and into another containing an appropriate amount of dried NaCl. By this procedure, both samples had identical antigen–antibody ratios and identical levels of tritium. In one case, Fab′ would exchange in reacted (species IV, Figure 1), the other unreacted (species I). Exchange out was accomplished by first making each sample 20 mM in EDTA before gel filtration chromatography on gels equilibrated with 10 mM EDTA. Thus, any tritium carried by Glu-Ala-Tyr would exchange out on the column. For the sample which was exchanged in unreacted, species I, further exchange out was done with reaction, species II. For the sample which was exchanged in reacted, species IV, further exchange out was done unreacted, species VI. By this manipulation, the effects of combining-site hydrogens are kept constant, since in one case, species I → species II, they exchange in and are prevented from exchanging out whereas in the other case, species IV → VI, they are prevented from exchanging in. If ligand binding effects are restricted to the combining site, then the exchange-out curves for these experiments should remain a constant difference apart over the exchange-out period (see Figure 1). That difference should correspond to the number of amide hydrogens which are combining-site associated.

The results of such an experiment on Fab′ with intact disulfide bonds are depicted by the two upper curves of Figure 3. From the differences in hydrogens per molecule of the curves, it is clear that these curves are not parallel. At the start of exchange out, there is a difference of 10 hydrogens

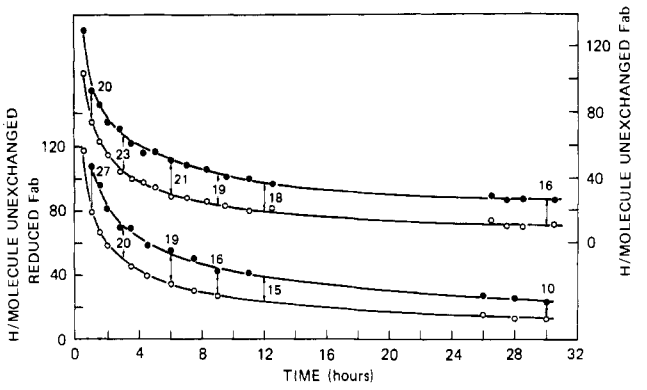

FIGURE 3: Exchange-out curves using the experimental protocols (species I → species II vs. species IV → species VI) for native or reduced anti-GAT–Ca^{2+} Fab′ at pH 6.0. Fab′ was exchanged in at 100 mCi/mL, 4 °C, for 19 h in Cac buffer, pH 6.0, in the presence of 20 times excess Glu-Ala-Tyr; species I → species II (●), species IV → species VI (○). Exchange out was initiated on a Bio-Gel P-2 column immediately following addition of 10 mM EDTA to exchanged-in samples. The top curves are for native Fab′ with the ΔH/molecule at time zero [species I_{t_0} – species IV_{t_0}] equal to 10 hydrogens. The bottom curves are reduced and alkylated Fab′ done by the same protocol.

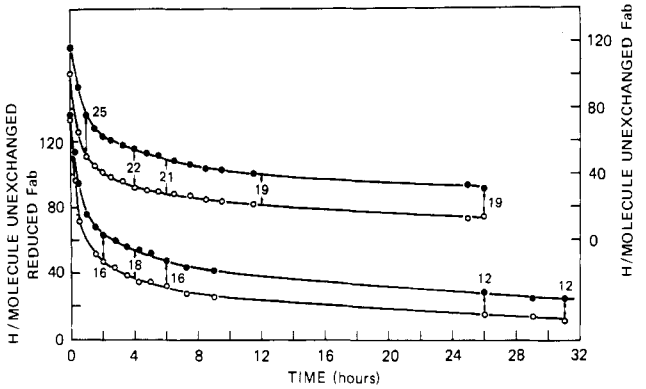

FIGURE 4: Exchange-out curves using the experimental protocols (species I → species II vs. species IV → species VI) for native or reduced anti-GAT–Ca^{2+} Fab′ at pH 7.8. The experimental procedure was identical with that described in the legend to Figure 3, except exchange in and exchange out were done at pH 7.8; species I → species II (●), species IV → species VI (○). The top curves are native Fab′ with ΔH/molecule at time zero equal to 17 hydrogens. The bottom curves are reduced and alkylated Fab′ with $\Delta H/\text{molecule}_{t=0}$ equal to 2.5 hydrogens.

per molecule, which reaches a maximum of 23 hydrogens per molecule between 2 and 4 h and becomes essentially a constant difference of 16 hydrogens per molecule after 14 h or so. As discussed above, this final difference of 16 amide hydrogens corresponds to the number of combining-site hydrogens which can be prevented from exchange when the exchange out is done by this protocol. An identical experiment, the lower two curves of Figure 3, shows the effect of total reduction and alkylation of all Fab′ disulfides. From the figure, it is clear that these two curves, like those of nonreduced Fab′, are also nonparallel. It appears that the major difference between native and reduced Fab′ is that the number of combining-site amide hydrogens which are ligand affected is lowered in the case of reduced Fab′ by six. There also appears to be a forward time shift where the maximum occurs. Although the experiments depicted were done on sheep Fab′ qualitatively, identical results were obtained with rabbit antibody.

The results of experiments identical in protocol, except done at pH 7.8, are shown in Figure 4. The upper set of curves is for native Fab′, the lower for the totally reduced and alkylated molecule. From the curves and the difference values

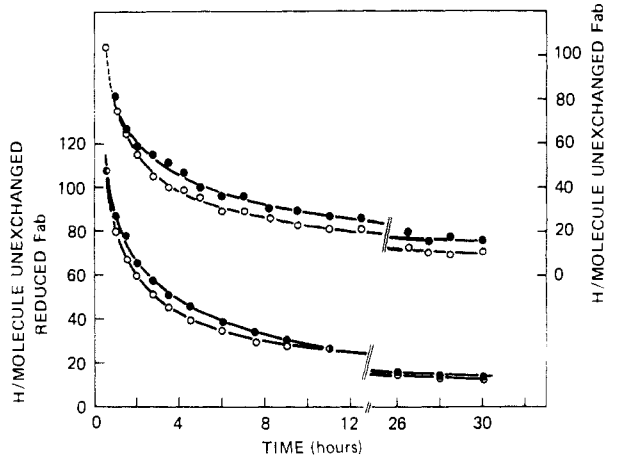

FIGURE 5: Exchange-out curves using the experimental protocols (species IV → species V vs. species IV → species VI) for native or reduced Fab′ at pH 6.0. Fab′ was exchanged in with ligand reaction (species IV) at pH 6.0, 4 °C, for 19 h with 20 times excess Glu-Ala-Tyr. The top curves are the exchange out of native Fab′, and the bottom curves are reduced and alkylated Fab′, either with ligand reaction, species V (●), or with no ligand reaction, species VI (○).

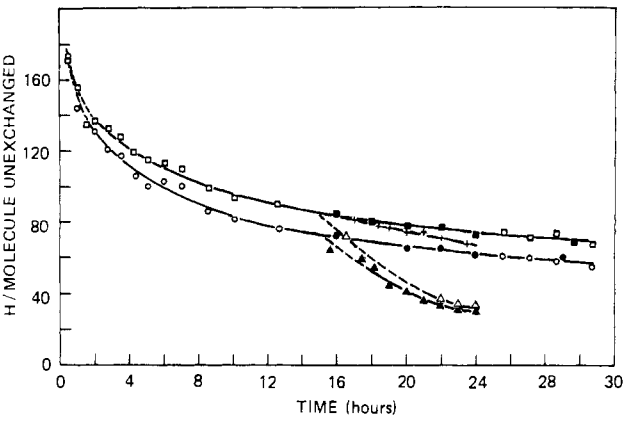

FIGURE 6: Exchange-out curves using the experimental protocols (species IV → species V vs. species IV → species VI) for native anti-GAT–Ca^{2+} Fab′ at pH 6.0 with crossover to pH 7.8 at 15 h. Fab′ was exchanged in with ligand reaction (species IV) at pH 7.8, 4 °C, with 20 times Glu-Ala-Tyr excess for 19 h. Samples were exchanged out at pH 6.0 with ligand reaction, species V (□, ■), and without ligand reaction, species VI (○, ●). The pH 7.8 crossover into Ca^{2+}–Tris (△) or EDTA–Tris (▲) was initiated at 15 h. Reacting Fab′, species V (□, ■), was crossed into EDTA–Cac (species VI) at 15 h (+). This figure is a composite of two separate experiments, data points ○, □, and + and ■, ●, △, and ▲.

given, it is clear that both sets of curves have nonparallel behavior similar to those done at pH 6.0. For the native molecule, a zero time difference of 17 hydrogens was obtained. This value reaches a maximum of 25 hydrogens per molecule somewhere between 1 and 2.5 h. This is in contrast to the maximum observed at pH 6 which occurs between 2 and 4 h. From 12 h onward, the curves became parallel at a constant difference of 19 hydrogens per molecule, corresponding to the number of combining-site hydrogens affected in this protocol. In the case of reduced Fab′, the curves exhibit a maximum difference at pH 7.8 somewhere before 2 h as was the case at pH 6.0. Just as was the case at pH 6.0, total reduction affects the number of combining-site hydrogens, lowering that number by seven.

Since the protocols used in the experiments of Figures 3 and 4 have hydrogens exchanging into non-combining-site regions of Fab′ under two sets of conditions, i.e., reacted (species IV) and unreacted (species I), it was of interest to determine if differences in non-combining-site hydrogens would be observed for reaction and nonreaction when exchanged-in hydrogens are identical. For accomplishment of this in the absence of unlabeled combining-site hydrogens, the following protocol was used. Fab′ was exchanged in in the presence of 20 times excess of Glu-Ala-Tyr in 10 mM calcium–Cac, pH 6.0 (species IV). After 19 h of exchange in at 5 °C, the sample was made 20 mM in EDTA, split in two, and chromatographed on Bio-Gel columns equilibrated with 10 mM EDTA–Cac. One sample was collected in an amount of calcium–Cac buffer to achieve 20 mM final calcium concentration, and other in an equivalent amount of EDTA–Cac. Exchange out was continued in 10 mM Ca^{2+} and EDTA, respectively, by rapid dialysis. By this protocol, only hydrogens which can exchange into Fab′ away from the combining site and under conditions of reaction are examined. Figure 5 shows typical results of experiments on native or reduced Fab′ done at pH 6.0. For both pairs of curves, the upper curve corresponds to Fab′ which is ligand reacted during exchange out; the lower curves correspond to the unreacted molecule. It can be seen that for native Fab′ reaction retards about six hydrogens from exchanging. This difference is maintained for at least 30 h. In the case of totally reduced Fab′, the curves diverge apart, reaching a maximum difference somewhere between 3.5 and 6.5 h, and have nearly approached each other by 11 h. By 30 h, however, a small and reproducible difference does exist. When identical experiments were done at pH 7.8, curves similar to those for reduced Fab′ were obtained (lower curves, Figure 5), except that for native Fab′ about half of the number of hydrogens were involved and in the case of reduced Fab′ a small, but perceptible, separation of the curves occurred between 2 and 5 h.

When unliganded Fab′ was exchanged in with or without Glu-Ala-Tyr present at pH 7.8 and exchanged out at pH 6.0, the separation between hydrogen exchange curves for the reaction and no reaction conditions was nearly identical with that of Figure 2. This indicates that combining-site amide hydrogens are unaffected in magnitude by the pH shift. [Similar results have been obtained for calcium-dependent antibodies to human serum albumin (M. Frankel and P. A. Liberti, unpublished experiments).] To determine if this type of pH change has any effects on the apparent non-combining-site hydrogens described above, an experiment identical with that of Figure 5 was performed except that Fab′ was exchanged out at pH 6.0 with reaction and the absence of reaction. The results for this experiment are shown in Figure 6. As was the case for experiments done totally at pH 7.8 or at pH 6.0, there is an apparent retardation of amide hydrogens for the reacted exchanging-out Fab′ compared with no reaction. As can be seen, there is involvement of about 12 hydrogens. Also shown in the figure is the result of an EDTA crossover initiated at 15 h, which was done by taking liganded Fab′ and dissociating the complex in EDTA, thus continuing exchange out in the absence of reaction. This manipulation allows estimation of the half-time of affected hydrogens. From the manner in which the reaction curve approaches the no-reaction curve, a half-time of exchange for the amide hydrogens involved can be estimated at 8–10 h. When this aspect of the experiment was repeated, an estimate of half-time of exchange for these hydrogens was 9–10 h, indicating that the affected hydrogens are slow-exchanging hydrogens at pH 6.0.

Since the affected hydrogens had exchanged into Fab′ at pH 7.8 under reacting conditions, it was of interest to show that they could exchange out at pH 7.8 when Fab′ is liganded. Accordingly, a cross-over experiment into pH 7.8 buffer was initiated after 15 h of exchange at pH 6.0. The results of the

Table I: Effects of Antigen–Fab′ Reaction on Structural Amide Fab′ Hydrogens

group	exchange-in conditions[a]	ΔH/ molecule (reaction − control) at 24 h	$t_{1/2}$ (h) of ligand-affected hydrogens[b]
I	Fab: 19 h, 4°C 4 h, 45 °C	4 ± 1	5 ± 0.6
II	Fab: 17 h, 4 °C 6 h, 45 °C	6 ± 1	5 ± 0.6
III	reduced Fab: 20 h, 4 °C 2 h, 45 °C	3 ± 1	5 ± 0.6
IV	reduced Fab: 16 h, 4 °C 6 h, 45 °C	6 ± 1	4.5 ± 0.5

[a] Exchange-out conditions: 5 °C, pH 6.0, 20 times antigen excess. Control samples were exchanged out in the absence of antigen–antibody reaction throughout the course of the experiments, 24 h. Reaction samples were exchanged out for 15 h with no antigen–antibody reaction and from 15 to 24 h in the presence of 10 mM Ca^{2+}, i.e., with reaction taking place. [b] Estimated from exchange curves of reacting and control Fab samples.

cross-over curves for reacted Fab′ and nonreacted Fab′ at pH 7.8 are also shown in Figure 6. These curves fall below the pH 6.0 exchange-out curves rapidly and become nearly coincident by 9 h after exchange at pH 7.8. From the curves, it can be estimated that half of these hydrogens have exchanged out 3 h after initiation of the pH crossover.

When nearly identical experiments were performed on fully reduced Fab′, the results were only moderately affected, indicating that these additional hydrogens observed by exchanging in at the higher pH and out at the lower pH have little disulfide dependence, which was not the case for those hydrogens affected when exchange in and out were done at a single pH.

To show by another experimental route that structural amide Fab′ hydrogens are affected by Fab′–antigen reaction, Fab′ was exchanged in under conditions previously determined to label greater numbers of Fab′ hydrogens and yet retain antigen binding activity. Such Fab′ samples were then exchanged out for 15 h at pH 6.0 in the presence of 20 times Glu-Ala-Tyr excess but in the absence of antigen–antibody reaction. In this manner, the faster exchanging amide hydrogens as well as the combining-site hydrogens would exchange out. At 15 h after exchange out, a portion of the sample was crossed over into buffer containing 10 mM calcium to initiate reaction. Hydrogens per molecule remaining unexchanged were measured hourly to a total of 24 h. Table I shows the ligand-affected amide hydrogens obtained by this procedure. As can be seen for native Fab′ and for reduced Fab′, as many as six amide hydrogens are retarded from exchange when the more vigorous exchange-in procedures are used. In all instances, the affected hydrogens had half-times of about 5 h, indicating that the affected hydrogens are neither in magnitude nor in structure disulfide related.

Discussion

In preliminary experiments with these protocols, it became evident that a variety of ligand-induced changes in Fab′ might be occurring. Thus, it was felt that by comparing results at two different pHs and on Fab′ fully reduced or native effects apparent by one protocol might be distinguishable from another. Unreduced rabbit or sheep Fab′ had 1.2 and 1.7 iodoacetamide groups per molecule and migrated as a single entity in $NaDodSO_4$–polyacrylamide gel electrophoresis whereas reduced and alkylated Fab′ had 8 iodoacetamide groups per molecule and migrated as L and Fd chains in $NaDodSO_4$ electrophoresis.

A summary of ligand-induced effects on Fab′ is given in Table II. The numbers of amide hydrogens affected in each case, estimated half-times of exchange for affected hydrogens, and disulfide and pH sensitivity are listed. From the values of amide hydrogens, the number of hydrogens involved in each case is on the whole generally small. With the assumption that each represents a different group, a total of about 50 amide hydrogens are conformationally sensitive (as will be seen later, some values may be minimal estimates). These summed effects represent about 10% of the total amide hydrogens of Fab′, and by consideration of the fact that a combining site represents approximately 4% of Fab, 10% may be more significant than is at first apparent.

For combining site associated residues, the magnitude of ligand-affected amide hydrogens does not seem to be different for experiments done totally at pH 6.0 or 7.8, or exchanged in at pH 7.8 and out at pH 6.0 (Table II). The small differences observed are probably due to time effects related to the starting of the antigen–antibody reaction after exchange out commences. This has been observed before and seems to be dependent on some fast exchanging hydrogens associated with the combining site. For studies done on calcium-dependent antibodies to human serum albumin, the numbers of combining-site hydrogens affected by ligand interaction were also independent of pH (M. Frankel and P. A. Liberti, unpublished experiments).

At either pH, it appears that complete reduction of Fab′ disulfide bonds reduces the number of combining site associated residues which are ligand affected. This was somewhat surprising since reduced preparations fully bind to adsorbants. In addition to indicating the sensitivity of hydrogen exchange, this result suggests that disulfide bonds play some role in maintaining the integrity of the combining site or that iodoacetamide inclusion moderately distorts Fab′. In studies of idiotype–antiidiotype reactions, Harboe and colleagues (Harboe et al., 1969; Solheim et al., 1971) have found that an idiotypic determinant is completely lost upon reduction and

Table II: Parameters of Ligand-Affected Fab′ Amide Hydrogens

group	Fab′ region	no. of ligand-affected hydrogens: native Fab′	no. of ligand-affected hydrogens: reduced Fab′	pH sensitivity[a] (+/−)	$t_{1/2}$ (h)[b]
I[c]	combining site	16–19	10–12	−	3.2
II[d]	unknown	6–7 (17–47)[h]	6–7 (17–47)[h]	+	(2 → 3/1 → 6)[h]
III[e]	noncombining site	6	<6	+	
IV[f]	noncombining site	12	6	+	~8
V[g]	noncombining site	6	6	ND	>5

[a] pH sensitivity refers to a comparison of the numbers of ligand-affected amide hydrogens observed at pH 6.0 or 7.8. [b] $t_{1/2}$ values are for unliganded native Fab′, except for group II which represents a rate shift for unliganded to liganded Fab′ (see Discussion). [c] See Figures 2–4. [d] See Figures 2–4. [e] See Figure 5 and Results. [f] See Figure 6 and Results. [g] See Table I. [h] These values are probable estimates for group II hydrogens experimentally observed: see Discussion.

Table III: Magnitudes and Times of Occurrence of Maxima Generated by Rate Shifts of an Exchange Class to Longer Half-Times[a]

kinetic shift		maximum value of exponential difference term	time maximum difference occurs after exchange out (h)
unreacted $t_{1/2}$ (h)	reacted $t_{1/2}$ (h)	$(e^{-\lambda_r t} - e^{-\lambda_u t})$	
1	2	0.249[b]	2.0
1	3	0.348	2.5
1	4	0.471	2.5
1	5	0.534	3.0
1	6	0.582	3.0
2	3	0.148	3.5
2	4	0.249	4.0
2	5	0.325	4.5
2	6	0.384	4.5-5.0
3	4	0.105	5.0
3	5	0.185	5.0-6.0
3	6	0.249	5.5-6.5
4	5	0.081	6.0-7.0
4	6	0.148	7
5	6	0.066	7.0-9.0

[a] Values were generated iteratively from eq 1 and a computer program. [b] For a fully exchanged-in class of size C exchanging out at two different rates, these values times C give the maximum difference in H per molecule the curves will diverge.

alkylation of the heavy- and light-chain disulfide bond in a Waldenström macroglobulin. By careful immunochemical analysis, they were able to show that this determinant is clearly in the variable region, indicating that the intrachain disulfide which is in the constant region of Fab′ can affect variable region conformation. Although further exchange studies are required to ascribe our results to those particular bonds, it appears that our finding generally agrees with theirs.

The seven amide hydrogens involved in the maximum differences observed for curves generated by comparing species I → II vs. species IV → VI protocols (Figures 3 and 4) are most probably due to hydrogens which exchange slower in liganded Fab′ than in the unreacted molecule. This can be seen by considering that for the experiments on native Fab′ at pH 6.0 and pH 7.8 reproducible zero time differences were obtained. In both cases, the zero time difference values are very close to differences obtained at long times (17 start, 19 end at pH 7.8; 10 start, 16 end at pH 6.0). Thus, it seems reasonable that at the start of exchange out (species I and IV) the class of hydrogens involved in producing the maximum has totally exchanged in, i.e., whether Fab is exchanged in reacted or unreacted. If that is so, then for exchange out, some class of size C would be exchanging out under two different conditions. With the assumption of the involvement of a single class, that difference can be written

$$\Delta H = Ce^{-\lambda_r t} - Ce^{-\lambda_u t} = C(e^{-\lambda_r t} - e^{-\lambda_u t}) \qquad (1)$$

where λ_r and λ_u are rate constants for this class in the reacted and unreacted conditions. It can be shown that the term in parentheses goes through a maximum at some time after exchange out if λ_r is greater than λ_u and that the maximum depends on the magnitude of λ_r and λ_u as well as their ratios (Liberti et al., 1977).

We can obtain some further information about these hydrogens if, as suggested above, we assume class C is entirely exchanged in during the 18-h incubation. This places an upper boundary of 6 h ($3 \times t_{1/2}$) for $t_{1/2}$ of these hydrogens in the slower exchanging reacted state. Thus, by considering some possible shifts of λ_u to λ_r, we can generate some theoretical parameters. In Table III, times of exchange out when maxima occur for some possible reaction-rate shifts are tabulated. The magnitude of the term in parentheses of eq 1 associated with each shift is also given. Not immediately apparent from the table is that the difference exponential term increases and approaches unity when there is a dramatic change in magnitude as well as in the ratio of rates; also, such shifts result in poorly defined broad maxima (not shown). Choosing from the table the smallest and largest difference exponential terms commensurate with the maximum times observed experimentally (recall maxma occurred between 2 and 4 h after exchange out commenced for native Fab′ at pH 6.0) gives 0.148 for a 2–3-h shift and 0.582 for a 1–6-h shift (unliganded to liganded, respectively). Thus, from eq 1, the seven amide hydrogens experimentally observed could represent a class of amide hydrogens which could be anywhere in size between 17 and 47. With the present data, an unequivocal determination of the rate of constants involved as well as the numbers of hydrogens is not possible.

For the other experiments done in this manner, it appears that the class of hydrogens involved in the rate switch is in some way affected by Fab′ reduction and by pH. For example, the time of the maximum appears to occur earlier for the reduced molecule and for increased pH. On the basis of the above analysis, the fact that the magnitudes of hydrogens involved for both pHs are identical may or may not be significant.

Since the results for the preceding kinds of experiments suggest that affected non-combining-site hydrogens can entirely exchange into liganded Fab′, then it would be anticipated that Fab′ exchanged in reacted and exchanged out either liganded or unliganded should result in curves which start together, diverge apart, reach a maximum dfference sometime between 2 and 4 h, and eventually come together again. This expectation was observed experimentally for reduced Fab′ at pH 6.0 (Figure 5, lower curves) and for native or reduced Fab′ at pH 7.8 (not shown). The curves on native Fab′ at pH 6.0 (Figure 5, upper curves), on the other hand, do not merge at long times, suggesting that other effects are operative. For the pH 6.0 result on native Fab′, six hydrogens do not appear to exchange out in liganded Fab′. When an identical experiment was done except with exchange in taking place at pH 7.8, about 12 hydrogens were found to be involved (Figure 6). From the corresponding cross-over curve, these hydrogens require 8–10 h for half of them to exchange out at pH 6.0, suggesting they are structural amide hydrogens. From the cross-over curve into higher pH (pH 6.0–7.8), it appears that some of these affected hydrogens are pH sensitive. This latter behavior is in contrast to that noted above for combining-site hydrogens examined at each of these pHs. In addition, we have found that combining-site hydrogens are not sensitive to the kind of pH crossover done here (P. A. Liberti and M. Chu, unpublished experiments).

The origin of these hydrogens is not clear. Because exchange in was done in the presence of excess antigen and calcium, they cannot be combining site associated amide hydrogens. Oddly enough, these hydrogens exchange into reacted antibody, yet they apparently do not exchange out of reacted antibody. In addition, from Figures 5 and 6 it is clear that they exchange out of unliganded antibody, yet we have no evidence of their ability to exchange into unreacted antibody. This is clearly a paradox since exchange in and exchange out of a protein in a given state are governed by the same parameters. From our ability to reproduce these results repeatedly to a precision of ±2H/Fab and even by variation of a variety of parameters, we believe them to be free of artifacts. That being the case, our only alternative is to suggest that Fab exchanging in reacted is not totally equivalent to exchanging

out reacted and that this difference has some pH and disulfide dependence. The only possibility we can offer which makes that suggestion tenable is that exchange in is done at Fab concentrations of 5–7 mg/mL while exchange out is done better than 2 orders of magnitude more dilute. Our results imply that Fab–Fab interactions are concentration dependent and are also dependent on whether Fab is liganded or unliganded. In regard to the pH dependence of some of these affected hydrogens, it appears that there may be a region in Fab′ away from the combining site which is not affected by ligand reaction at pH 7.8, but is ligand sensitive at pH 6.0.

The results of experiments involving higher levels of tritium labeling (Table I) also show that ligand affects the structural amide hydrogens of Fab′. These hydrogens, like those involved in the pH-shift experiments, were found to be unaffected by disulfide reduction and are slowly exchanging. Since labeling of these hydrogens required increased temperatures, it seems that they are not the same amide hydrogens observed in the pH-switch experiments.

Taken together, the results of our exchange experiments indicate that ligand binding of Fab′ is quite a complicated phenomenon and that a variety of conformational changes in Fab′ are apparent with ligand binding. These changes appear to involve relatively small portions of Fab′ and are consistent with the concept that ligand binding alters segmental breathing of Fab′. It appears that the most dramatic change occurs in amide hydrogens associated with the combining site which switch to an apparent nonbreathing condition upon ligand binding. It seems, however, that breathing reactions in other parts of Fab′ are affected and that significant changes in rate constants are involved, indicating tightening of the structure. The significance of these effects or the ability to correlate them with known biological function(s) of parts of Fab′ removed from the combining site cannot be evaluated at this time since to date no other physical measurements have been able to distinguish effects in different parts of the molecule nor is it clearly known if Fab′ contributes in any way to effector functions of IgG.

Despite the lack of information concerning the latter, these results would be consistent with the concept that combining-site filling might result in alterations in the Fc portion of IgG (allosteric model) since such changes would have to be transmitted through Fab′, and, therefore, measurable changes in Fab′ conformation away from the combining site would have to be apparent. This consistency would, however, have to be based on the assumption that a conformational change could be transmitted that far, and from the analysis of ligand effects in hemoglobin, that does not seem likely (Baldwin & Chothia, 1979). On the other hand, antigens can be orders of magnitude larger than O_2, and perhaps a comparison is unfair. In spite of that, on the basis of the magnitude of changes observed here, any effects transmitted to Fc would seem to be small. That possibility should not necessarily be confused with being insignificant. However, in view of the size of the effects seen here for Fab′ and with the assumption of commensurate changes in Fc, the experimental rigor which would have to be employed makes such searches seem almost fruitless. On a more optimistic note, the recent availability of hybridoma antibodies against defined antigenic determinants (Kohler & Milstein, 1975) coupled with X-ray crystallographic technology might provide a route for an unequivocal solution to this problem.

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