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Hapten-Induced Structural Changes in Rabbit Immunoglobulin G with Specifically Mercuriated Inter-Heavy-Chain Disulfide†

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ABSTRACT: Rabbit antipolyalanyl immunoglobulin G was specifically modified by insertion of a mercuric ion between the cysteinyl sulfurs of its reduced single inter-heavy-chain disulfide bond. This mercuriated derivative was used for investigation of the effect of binding a divalent specific hapten to the antibody. Nuclear magnetic resonance of $^{35}Cl^-$ exchanging between the mercury coordination sphere and the solvent was used to monitor the hapten-induced change in the environment of mercury in the protein. Upon binding the divalent hapten the ^{35}Cl line width was reduced. This spectral change was interpreted as hapten-induced alteration of steric relations within the hinge region. The measurements of the circularly polarized component of protein tryptophyl fluorescence (CPL) revealed no difference between the native and mercuriated protein, but reduced and alkylated species had distinctly different CPL, especially at longer wavelengths (350–370 nm). A pronounced change in the CPL induced by binding the divalent hapten was apparent below 350 nm and was of identical magnitude for the native and mercuriated protein yet rather small for the reduced and alkylated derivative. In agreement with earlier studies it is proposed that fluorophores emitting below 350 nm are probably related to the binding site, while those emitting above 350 nm are more sensitive to other domains of the protein. The interdependence of the intactness of the hinge disulfide and the chirality of fluorophores within the binding site was demonstrated.

Binding of antigenic determinants to their specific antibodies has been shown to cause spectroscopic changes which were assigned to structural transitions induced in the immunoglobulins. Several efforts have been made to correlate these spectroscopic changes with the induction of effector functions of these antibodies (Schlessinger et al., 1975; Pecht, 1976; Pecht et al., 1977). One prominent feature which emerged from those studies is the strict requirement for the intactness of the inter-heavy-chain disulfide bonds(s) both for the induction of spectroscopic transitions and for the complement activation (Schur & Christian, 1964; Press, 1975). The single inter-heavy-chain disulfide bridge in rabbit IgG occupies a central structural position in the hinge region. Thus, it is expected that changes in the relation between the Fab and Fc domains would be sensed in this region. These relations range from the spatial disposition of the domains and their segmental flexibility to potentially more subtle structural changes within them as affected by binding mono- and polyvalent antigenic determinants.

A specific insertion of a probe into this disulfide group provides a unique possibility of investigating these interactions. Such a probe is a metal ion of high affinity for sulfhydryl residues, e.g., mercuric ions. One major advantage of this probe is that it is expected to produce a well-defined and rather mild perturbation in the IgG.

Mercuric ions have been shown to provide for a potent magnetic relaxation mechanism of halide nuclei possessing a quadrupolar moment (Stengle & Baldeschwieler, 1966; Haugland et al., 1967). We monitored the hapten-induced changes in the environment of mercury by the nuclear magnetic relaxation rates of $^{35}Cl^-$ exchanging between the metal coordination sphere and the bulk solvent. Possible concomitant changes within the protein domains were investigated by measurements of the circularly polarized emission of tryptophan residues (Steinberg, 1975).

Materials and Methods

Specific antibodies against poly(D-alanyl) diphtheria toxoid were raised in rabbits as described previously (Licht et al., 1971) and purified on a Sepharose-poly(D-alanyl)-RNase affinity column. Nonspecific rabbit IgG was obtained from pooled normal serum by ammonium sulfate precipitation and DEAE-Sephadex ion exchange chromatography (Licht et al., 1971).

The divalent hapten 1,7-diaminoheptanebis(tri-D-alanine) (bis-triala) was synthesized as will be described elsewhere (A. Licht, A. Gafni, and I. Pecht, unpublished results).

All the chemicals were of the highest commercial purity grade. Guanidine hydrochloride was "ultrapure" grade of Schwarz/Mann. Sephadex G-100 superfine (Pharmacia) and

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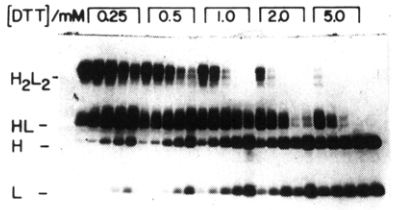

FIGURE 1: Distribution of reduction products of normal rabbit IgG as function of dithiothreitol (DTT) concentration and time for an 8-mg/mL protein solution, pH 8.0. For each DTT concentration (as marked in the figure) reduction was stopped by iodoacetamide after 5, 10, 20, 40, and 80 min (from left to right). The gel is a sodium dodecyl sulfate nonreducing polyacrylamide gradient from 5 to 20% (Laemmli, 1970).

Bio-Gel P30 (Bio-Rad) were used for gel filtration. Radioactive mercury was obtained as $^{203}HgCl_2$ from Amersham. Polyalanylated RNase was radioiodinated by the lactoperoxidase procedure (Marchalonis, 1969) using carrier-free $Na^{125}I$ (Amersham). Radioactivity was measured on a Packard automatic γ counter.

Protein concentrations were determined spectrophotometrically using an $E_{280nm}^{1\%}$ value of 14.0 for rabbit IgG. Antigen binding capacity of the native and modified antibodies was determined using ^{125}I-labeled polyalanylated RNase as antigen. The antigen–antibody complex was precipitated by *Staphylococcus aureus* carrying protein A (Brunda et al., 1977).

Analytical ultracentrifuge studies were performed using a Beckman Spinco Model E equipped with an optical UV scanner and operating at 25 °C and 52 000 rpm. Circular polarization of luminescence (CPL) was measured under identical conditions as before (Schlessinger et al., 1975) using an apparatus constructed at The Weizmann Institute of Science (Steinberg & Gafni, 1972). Nuclear magnetic resonance of ^{35}Cl was measured in the Fourier transform mode using a Bruker WH 90 spectrometer operating at 8.82 MHz with an internal deuterium lock. For each spectrum 12 500 pulses were accumulated with an acquisition time of 0.87 s.

Results

Reduction of the Inter-Heavy-Chain Disulfide. In order to optimize the yield of specifically reduced half-molecules (HL) (Palmer & Nisonoff, 1964) we followed the distribution of reduction products as a function of reductant concentration and reduction duration by polyacrylamide gel electrophoresis in sodium dodecyl sulfate in the absence of reducing agents (Laemmli, 1970). The reaction was stopped by the addition of excess iodoacetamide. The results of such an experiment performed at room temperature with normal rabbit IgG (8 mg/mL of protein in 0.1 M Tris, 1 mM EDTA, pH 8.0) are depicted in Figure 1. The optimal conditions chosen for further isolation of HL were 0.25 mM dithiothreitol and 40 min as judged from the favorable distribution of resultant molecular species. Since it appeared simpler to separate HL from the nonreduced IgG than from the heavy chain, we chose reducing conditions where most of the protein was either intact or HL.

The antipoly(Ala) antibody was reduced under the above described conditions. Reduction was terminated by dropwise addition of concentrated propionic acid to a concentration of 1 M. The resultant solution was applied to a Sephadex G-100 superfine column (2.9 × 100 cm) equilibrated in 1 M propionic acid containing 1 mM EDTA and run at a flow rate of 3 mL cm^{-2} h^{-1}. A typical elution profile is shown in Figure 2. The

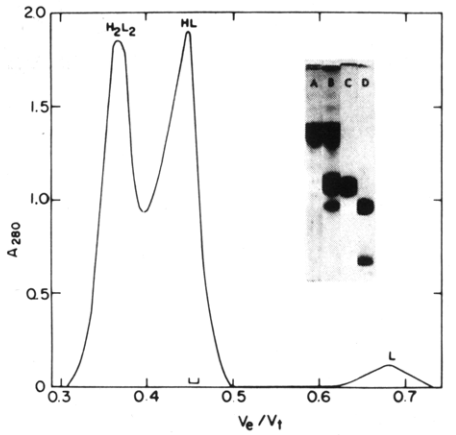

FIGURE 2: The elution profile of the reduction mixture applied to a Sephadex G-100 superfine column (2.9 × 100 cm) equilibrated with 1 M propionic acid and 1 mM EDTA. H_2L_2 denotes native IgG; HL, the reduced half-molecule; and L, the light chain. Inset: 5.6% sodium dodecyl sulfate nonreducing polyacrylamide gels (Fairbanks et al., 1971) of the native IgG (A), reduction mixture after 40-min reduction by 0.25 mM DTT (B), separated HL species from the HL peak (C; fractions marked by ⊔), and fully reduced IgG (D).

left peak ($V_e/V_t = 0.37$) represents the void volume containing the nonreduced protein. The peak to the right ($V_e/V_t = 0.44$) consists of HL with traces of the free heavy chain in its descending part. Light chain was eluted at $V_e/V_t = 0.68$.

Reconstitution and Mercuriation. The fractions from the HL-containing peak were pooled and diluted 10-fold with doubly distilled water. The pH was subsequently raised to 4.0 with NaOH. After 2 min 0.1 M $HgCl_2$ was added to make the solution 1 mM in mercuric ions (Steiner & Blumberg, 1971; Sperling et al., 1969). This concentration was sufficient to saturate the SH groups and EDTA in the solution. The excess of mercuric ions was removed by gel filtration on a Bio-Gel P-30 column (1.0 × 50 cm) equilibrated in 0.01 M phosphate, 0.15 M NaCl, pH 7.0.

Using radioactive $^{203}HgCl_2$ the final incorporation of 0.95 ± 0.20 mercuric ions per molecule of IgG was established. The extent of incorporation was not affected by the presence of the divalent hapten bis-triala. If instead of mercuric chloride the sodium chloride was added, the protein fully reoxidized. This reoxidized species was shown to bind no more than 0.15 mercuric ions per molecule. An analysis of the antigen binding capacities of the three forms of the protein for polyalanylated RNase (Brunda et al., 1977) revealed no significant differences.

Ultracentrifuge Studies. In order to assess the strength of the mercuriated inter-heavy-chain disulfide bond we titrated the reduced and alkylated, mercuriated, and native proteins with guanidine hydrochloride (Lapanje & Dorrington, 1973) in the ultracentrifuge. The $s_{20,w}$ values were calculated using relative viscosities and solvent densities given by Kawahara & Tanford (1966), assuming for all the concentrations of guanidine hydrochloride a partial specific volume of the protein $\bar{V} = 0.72$ cm^3/g. The $s_{20,w}$ values of native, mercuriated, and reduced and alkylated derivatives at 1.4 M GuHCl were 6.37, 6.45, and 6.47, respectively. The corresponding values at 1.8 M GuHCl were 6.46, 5.67, and 5.54. Clearly, both reduced and alkylated as well as the mercuriated species begin to dissociate between 1.4 and 1.8 M guanidine hydrochloride. For the former derivative these results agree well with the data of Lapanje & Dorrington (1973).

Circular Polarization of Luminescence. We have performed measurements of the circularly polarized fluorescence of native, selectively mercuriated, and reduced and alkylated antibodies

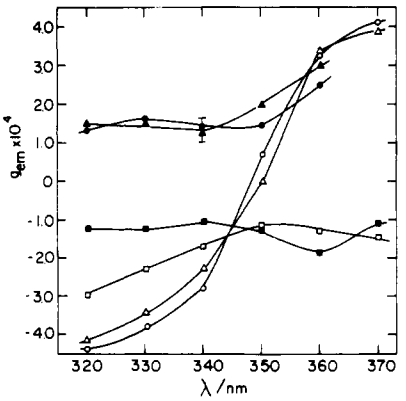

FIGURE 3: Circular polarized fluorescence spectra of the hapten-free native (○), mercuriated (△), and reduced and alkylated (□) anti-polyalanine IgG. Closed symbols: spectra of the respective protein derivatives in the presence of a twofold molar excess of divalent hapten bis-triala. Buffer: 0.15 M NaCl, 10 mM phosphate, pH 7.4. The bar indicates the estimated error range.

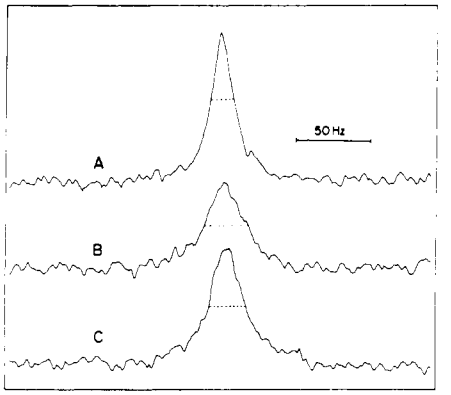

FIGURE 4: The ^{35}Cl nuclear magnetic resonance spectra of 0.5 M Cl^- solution containing native rabbit antipolyalanine IgG (A), mercuriated protein (B), and mercuriated derivative with bis-triala hapten in a 1.75-fold molar excess over the antibody (C). Buffer: 0.5 M NaCl, 10 mM phosphate, pH 7.4.

and of their complexes with the divalent hapten bis-triala. The variation of the anisotropy factor ($g_{em} = \Delta f/2f$, where Δf is the intensity of the circularly polarized component and f is the total fluorescence intensity at a given wavelength) with the wavelength is caused by different degrees of asymmetry of the environment of the fluorophores emitting at different wavelengths. The open symbols in Figure 3 denote the hapten-free proteins. It is important to note that there is no difference in g_{em} between the native protein and its mercuriated derivative (circles and triangles, Figure 3). The value of g_{em} up to around 350 nm becomes positive upon binding of divalent hapten (2:1 molar ratio of hapten/antibody), while above this wavelength it remains virtually unaffected. On the other hand, reduction and alkylation of the free protein cause a more negative value of g_{em} than in the other two derivatives above 350 nm. Addition of the divalent hapten is reflected primarily in the blue part of the spectrum, although the magnitude of the induced change is markedly smaller than that observed for the intact or mercuriated antibodies. Therefore, it seems that binding of the divalent hapten to these antipolyalanyl antibodies is expressed in spectral change mainly below 350 nm.

Enhancement of Nuclear Magnetic Relaxation of ^{35}Cl. It has been shown by Stengle & Baldeschwieler (1966) that nuclear magnetic resonance spectra of halide ions in the solvent may be used to probe the accessibility of mercury inserted into proteins. Since the original suggestion, a number of biochemical studies along these lines have been carried out (cf. Dwek, 1973).

In an aqueous solution a chloride ion is symmetrically solvated and the asymmetry of the electric field gradient at its nucleus is negligible. As the chloride enters the coordination sphere of the mercury atom, this symmetry is distorted and the orientation and magnitude of the electric field gradient fluctuates, providing a potent and specific relaxation mechanism (Stengle & Baldeschwieler, 1966). Since ^{35}Cl has a quadrupolar nucleus the fast-exchange mechanism limit between mercury bound and bulk chloride pertains, and thus a single ^{35}Cl NMR signal is observed, contributed by both magnetic sites (Luz & Meiboom, 1964). The line width will be the population-weighted average of the narrow free-chloride line and the significantly broader line due to the bound chloride; consequently the mercury-enhanced ^{35}Cl half-line width, $\Delta\nu_{Hg}$ (in Hz), is

$$\Delta\nu_{Hg} = P_M q K (e^2qQ)^2 \tau_c \qquad (1)$$

where P_M is the mole fraction of ligand nuclei bound to the mercury, q is the number of "coordination" sites available at any particular mercury for chloride exchange, K is a constant comprising nuclear spin and asymmetry parameters, e^2qQ is the quadrupole coupling constant in Hz, and τ_c is the effective correlation time. For a more detailed elaboration of the mechanism of enhancement of relaxation of quadrupolar nuclei the reader is referred to the original paper by Stengle & Baldeschwieler (1966).

In Figure 4 are depicted ^{35}Cl NMR spectra recorded at 8.82 MHz of the 0.5 M solution of NaCl containing native anti-poly(Ala) IgG [(A) in Figure 4], its mercuriated derivative (B), and mercuriated derivative containing bis-triala in the ratio 1:1.75 on a molar basis (C). In all the cases the protein concentration was kept strictly at 3.0 mg/mL. Each spectrum was obtained by the Fourier transformation of the average of 12 500 pulses.

A quantitative evaluation of these spectra in terms of quadrupole coupling constant, effective correlation time, and the actual coordination number is not possible here prior to the independent determination of two of these parameters. However, the existent data enable us to draw some structural conclusions based on the comparison of the spectra.

The value of 16 Hz for the ^{35}Cl line width in the solution of the native protein [(A) in Figure 4] is not much larger than that found in pure chloride solutions and agrees well with those found in other solutions of metal-free proteins (Stengle & Baldeschwieler, 1966; Dwek, 1973). The substantial broadening of the ^{35}Cl line [(B) in Figure 4] is somewhat smaller here than that usually observed in mercuriated proteins where mercury is attached to a single cysteine. This is due here to the fact that two coordination sites on the mercury are already occupied by cysteinyl sulfurs. Only weak binding sites remain available for chloride exchange, consistent with the recent X-ray studies of model complexes (Taylor & Carty, 1977). Also, some sterical constraints by the protein may impede chloride exchange, so that the time-averaged number of these anions at the coordination sites may be smaller than in analogous simpler mercury complexes. On the other hand, traces of free mercury in the solution, if present at all, would not be expected to affect the line widths, due to the very short rotational correlation times of such complexes (cf. eq 1).

At the concentrations of divalent hapten we used to obtain spectrum C (Figure 4) a range of soluble oligomeric complexes, composed primarily of dimers, is formed (A. Licht, A. Gafni, and I. Pecht, manuscript in preparation). The observed narrowing of the ^{35}Cl line is therefore not due to the decrease

of the concentration of mercuriated protein in the solution, which might occur upon precipitation.

Discussion

The specific insertion of a mercuric ion into the inter-heavy-chain disulfide of rabbit IgG results in the lengthening of this bridge by some 3 Å (Yakel & Hughes, 1954; Bradley & Kunchur, 1965). This small alteration neither affects the antigen binding capacity of the molecule nor does it cause structural perturbations resolvable by tryptophan circularly polarized emission measurements. The inserted mercuric ion is therefore a useful probe positioned in a most interesting location in the immunoglobulin structure.

By monitoring the NMR signal of $^{35}Cl^-$ exchanging between the mercury coordination sphere and the solvent we were able to detect changes in the environment of this probe upon binding divalent hapten to the antibody. There are several possible explanations for the bis(hapten)-induced narrowing of the $^{35}Cl^-$ NMR line. The prevalent effect is the decrease in the efficiency of the relaxation mechanisms. Not invoking changes in the quadrupole coupling constant, there are two parameters which may be dominant (eq 1). One of them is τ_c, the effective correlation time. It is dominated by the shorter of the two correlation times, the rotational time of the complex, τ_R, and the lifetime of the exchanging nucleus at the metal site, τ_M. Since at the employed antibody/divalent hapten ratio mostly dimers are formed (A. Licht, A. Gafni, and I. Pecht, manuscript in preparation), τ_R increases, and this should lead to a broadening of the NMR line. This fact shows that τ_M is at least commensurate with τ_R, if not shorter, and therefore enables us to set the lower limit for the exchange lifetime as $\tau_M < \tau_R$, i.e., less than 168 ns as measured by Yguerabide et al. (1970) for the value of τ_R.

The other parameter in eq 1 is $P_M q$, the fraction of the total relaxing species bound to the metal, i.e., the probability of finding the relaxing chloride nucleus at the metal site. It appears that the hapten-induced change of this probability term is the simplest explanation of the data. Namely, steric constraints in the vicinity of the mercury caused by changes in the dynamic spatial disposition within the IgG molecules forming hapten-bridged oligomers (A. Licht, A. Gafni, and I. Pecht, manuscript in preparation) will modulate Cl^- exchange rates and/or the time average of the number of effective coordination sites available for exchange. In view of the mutual interdependence of the steric (q in eq 1) and dynamic (τ_M and τ_c) parameters, a more detailed mechanistic interpretation is, however, premature. Still, the changes in solution viscosity or in protein molecular weight, resulting from hapten-induced oligomerization, cannot account for the narrowing of the ^{35}Cl NMR line as these effects would lead to line broadening.

CPL measurements monitor structural changes expressed in the chirality of tryptophan residues distributed throughout the IgG structure. Our study suggests that perturbation of fluorophores emitting at different parts of the emission band results from different molecular events occurring in the immunoglobulin. Below 350 nm the emitters which are in a more apolar environment exhibit strongly negative anisotropy. The "blue" region of the spectrum in both the native and mercuriated protein is particularly sensitive to the binding of bis(hapten). In the same spectral region of the reduced and alkylated derivative, binding the divalent hapten induces a change in the same direction, although of considerably smaller magnitude. In the longer wavelength part of the emission band, the fluorescence is mainly due to the more exposed residues. This "red" region is insensitive to hapten binding in all of the derivatives. In contrast, reduction and alkylation caused a marked change in this region, g_{em} becoming more negative. The same procedure has a rather limited effect on circular polarization in the blue region. A consistent finding in all antibody CPL studies to date has been that breaking the interchain disulfide bridge largely abolishes the hapten- or antigen-induced effect on the CPL spectrum. It is noteworthy that in the case of CPL studies on rabbit IgG antibodies of different specificities (Jaton et al., 1975; Pecht et al., 1977) reduction and alkylation did not per se result in a significant alteration in the red part of the CPL spectrum. Therefore if this phenomenon is indeed specificity dependent, it must arise at least in part from the perturbation of variable-region tryptophans. Since it has been demonstrated that reduction of the hinge disulfides leads to an increase in segmental flexibility as judged by physical (Venyaminov et al., 1976; Chan & Cathou, 1977) and biological measurements (Romans et al., 1977), it may be that some variable-region tryptophans of antipolyalanyl immunoglobulins are sensing change in their asymmetric environment brought about by increased segmental flexibility.

The role of an interchain disulfide bridge in modulating the conformational dynamics in immunoglobulin domains has recently been further illustrated by Ely et al. (1978). The intact disulfide between the two light chains of the Bence-Jones dimer Mcg prevented the reversibility of the hapten-induced structural changes.

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High Concentration Active Enzyme Centrifugation: Analysis of Active Polymeric Forms at up to 10000-Fold Higher Concentrations than with Conventional Methods†

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ABSTRACT: This paper describes the theoretical basis, experimental technique, and experimental evaluation of a new method of analysis called "high concentration active enzyme centrifugation". It extends by up to four orders of magnitude the upper concentration limits at which the technique of "active enzyme centrifugation" can be used for analysis of enzyme structure. This new theory is largely based on certain properties of Gaussian curves which we have described in previous publications [Wei, G. J., & Deal, W. C., Jr. (1976) *Anal. Biochem. 75*, 113–121; *Anal. Biochem.* (1978) *87*, 433–446]. One of the most important aspects of this development is that it extends the concentration range upward so that experiments can be performed on enzymes in the active polymeric forms corresponding to their in vivo states. Furthermore, this expansion includes the range in which most enzymes go through all their association–dissociation transitions from one polymeric form to another. Hence, the method can be used to define the various concentration-dependent transitions and also to ascertain which of the various polymeric forms of an enzyme are active, under various conditions. This method also retains the many favorable characteristics inherent in the active enzyme centrifugation technique. In studies with lactate dehydrogenase, the results from this method of *band* sedimentation were identical within experimental error (about 1.5%) with results from conventional *boundary* sedimentation velocity studies.

The method of "active enzyme centrifugation" (reacting enzyme centrifugation), a band sedimentation technique introduced by Cohen and co-workers (Cohen, 1963; Cohen & Mire 1971a,b), provides the unique capability of analyzing both enzyme structure and activity at the same time. This avoids hazardous extrapolations and correlations between activity properties measured with low enzyme concentrations and structural properties measured with high enzyme concentrations. This very powerful technique has a number of unique advantages over other conventional types of physical analysis of macromolecular structure. It has extreme sensitivity (requires only minute quantities (nanograms) of enzyme), analyzes the active form of the enzyme, and does not require purified enzyme. However, the extreme sensitivity of the technique provides not only one of its greatest and most unique advantages but also its greatest limitation; measurements above approximately 0.1 μg/mL are excluded for some enzymes with present methods of analysis (Cohen & Mire, 1971a).

Obviously, it would be of great interest to analyze the concentration region from 0.1 μg/mL up to 1000 μg/mL and higher, since this is the concentration region in which most enzymes exist in vivo and also the range in which most enzymes undergo shifts in their association–dissociation equilibria.

We have developed a theory for such analysis based largely on the theoretical properties of Gaussian curves described in previous publications (Wei & Deal, 1976, 1978). We have tested the theory with experiments using our computer-controlled ultracentrifuge scanner system (Wei & Deal, 1977; Wei, G. J., Deal, W. C., Jr., Gasper, E., & Johnson, C. S., unpublished results). This development removes any theoretical upper limits on the enzyme concentration which can

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