Use of *N*-Methylnicotinamide Chloride as a Conformational Probe in Proteins. Identification of the Binding Sites in Chicken Egg-White Lysozyme and a Comparison with Bovine α-Lactalbumin*

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ABSTRACT: The interaction site of a complex between *N*-methylnicotinamide chloride and chicken egg-white lysozyme has been analyzed by means of chemical modification and by competition with saccharide inhibitors. The charge-transfer transition between the indole moiety (donor) of a solvent-available tryptophan residue in lysozyme and *N*-methylnicotinamide (acceptor) was used to study the interactions. The competitive inhibitors *N*-acetylglucosamine and *N*-acetylglucosamine trimer compete for the *N*-methylnicotinamide binding site. As the concentration of inhibitor is increased, the apparent association constant for the nicotinamide–lysozyme complex decreases with no change in the apparent extinction coefficient, indicating that the nicotinamide interaction occurs in the active-site "cleft" of the protein molecule. Specific oxidation of Trp-62 by *N*-bromosuccinimide destroys the potential for formation of the native complex but, in addition, appears to make a second tryptophanyl residue available for complexation with reduced affinity. By use of partially oxidized derivatives it has been shown that the resulting titration can be fitted to a model in which *N*-methylnicotinamide binds to two separate molecular species with different binding constants. An alternative model, in which two sites are available on the native protein and oxidation destroys only one, is untenable in the light of presently available data. The physical parameters for the second site, not present in the native enzyme, closely fit the observed titration of bovine α-lactalbumin. These results are interpreted to mean that the primary site in native lysozyme is Trp-62. Further, it is suggested that the secondary site, available only in oxidized lysozyme and native α-lactalbumin, may be the indole moiety of Trp-63 in lysozyme (residue 60 in α-lactalbumin).

It has recently been demonstrated that chicken egg-white lysozyme forms a yellow intermolecular complex with *N*-methylnicotinamide chloride (Deranleau *et al.*, 1969).[1] By comparison with model studies (Deranleau and Schwyzer, 1970), a solvent-available tryptophan residue on the lysozyme molecule was implicated as the primary binding site for *N*-methylnicotinamide. The evidence for the assignment of a tryptophan residue as the binding site can be summarized briefly as follows. The appearance of the yellow color on addition of increasing amounts of *N*-methylnicotinamide chloride follows a hyperbolic binding curve (absorbance *vs.* *N*-methylnicotinamide concentration), characteristic of a single class of sites with an association constant, k, of 3.2 l./mole and an extinction coefficient, ϵ, of 1040 l./mole cm at 350 nm. These values are in good agreement with values obtained on complexes between *N*-methylnicotinamide and tryptophan or tryptophan analogs. Further, the absorption spectrum of the complex with lysozyme closely resembles the absorption spectra of the complexes with tryptophan and tryptophan analogs containing the indole nucleus.

In the case of the model complexes (Deranleau and Schwyzer, 1970), the position and intensity of the new absorption band appearing in the ultraviolet-visible region of the spectrum is consistent with the assignment of the band to a charge-transfer transition between the indole moiety (donor) and the nicotinamide positive ion (acceptor). From the spectral similarity of the *N*-methylnicotinamide complexes with tryptophan models and with lysozyme, it is virtually certain that a charge-transfer transition explains the appearance of the yellow color in the lysozyme complex (Deranleau *et al.*, 1969).

Examination of lysozyme models (Blake *et al.*, 1967b; Harte and Rupley, 1968) based on crystallographic studies (Blake *et al.*, 1967a,b) reveals that there are four potential indole ring binding sites for *N*-methylnicotinamide which are, in order of likelihood from steric considerations, Trp-62, -63, -123, and -108. Three of these residues lie in the inhibitor binding "cleft" of the lysozyme molecule (62, 63, and 108), and of the four, only Trp-62 appears to be more or less completely available for a maximum overlap, face-to-face interaction with *N*-methylnicotinamide. Utilizing published work on the chemical reactivity of tryptophan residues in lysozyme (Hayashi *et al.*, 1965; Hartdegen and Rupley, 1967) and on the binding of inhibitors of the enzymatic function of lysozyme (Blake *et al.*, 1967b; Rupley *et al.*, 1967), we have attempted a characterization of the specific *N*-methylnicotinamide binding site or sites. The information so obtained

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[1] And, in addition, inhibits the growth of "hiochi" bacteria in sake (Yajima *et al.*, 1968).

has been compared with the titration of α-lactalbumin for the purposes of further evaluating the proposed three-dimensional homology of these two proteins.

Experimental Section

Materials. Lysozyme (lot no. LY 644A) was purchased as a twice-recrystallized preparation from Worthington Biochemicals Corp. and used without further treatment. *N*-Methylnicotinamide iodide was prepared from methyl iodide and nicotinamide, and was converted into *N*-methylnicotinamide chloride by treatment with solid silver chloride as described by Karrer *et al.* (1936). All experiments were carried out with the same preparation of *N*-methylnicotinamide chloride (ε 0.01 (at 350 nm)).

α-Lactalbumin was prepared by the method of Gordon and Ziegler (1955) and recrystallized three times with ammonium sulfate after acid precipitation.[2] The final product was purified on a 2.0 × 100 cm column of G-100 (equilibrated in 0.1 M sodium phosphate, pH 6.5), dialyzed against distilled water, and lyophilized.

N-Acetylglucosamine (*N*-AcGlu)[3] was purchased from Mann, and *N*-acetylglucosamine trimer (tri-*N*-AcGlu) was a gift from Dr. John Rupley. *N*-Bromosuccinimide (NBS) was purchased from Eastman Organics, and *Micrococcus lysodeikticus* cells came from Worthington Biochemicals Corp. 2-Hydroxy-5-nitrobenzyl bromide (HNB) was obtained from Cyclo Chemical Corp. and recrystallized before use.

Methods. Titrations with *N*-methylnicotinamide were performed by the addition of solid reagent to 2.00 ml of a solution containing ~10 mg/ml of lysozyme in water. In each titration experiment, 10–15 aliquots of solid nicotinamide were added to a concentration of 1 M and the absorption spectrum was recorded from 500 to 300 nm on a Cary Model 15 recording spectrophotometer[4] after each addition. The data were analyzed as described previously (Deranleau, 1969; Deranleau *et al.*, 1969). Lysozyme concentrations were determined spectrophotometrically (based on an ε value of 37,913 l. mole^{-1} cm^{-1}) on aliquots removed from the stock solution.

Partially oxidized lysozyme was prepared as described by Hayashi *et al.* (1965). The NBS-oxidized product was recovered by lyophilization after extensive dialysis in $^{18}/_{32}$ Visking casing, which minimized losses during dialysis. The protein concentration of the partially oxidized samples was determined using the extinction coefficient of the fully oxidized sample (Hayashi *et al.*, 1965, adjusted for the extent of the reaction), and validated by amino analysis. The protein concentration of the α-lactalbumin solutions was calculated from an $E^{1\%}_{280}$ of 20.5 ± 0.4 (average of values given by Kronman and Andreotti, 1964, and Wetlaufer, 1961) assuming a molecular weight of 14,437 (Brew *et al.*, 1967).

Lysozyme activity was measured against suspensions of *M. lysodeikticus* cells as described previously (Bradshaw *et al.*, 1967). The amount of oxidation of the Trp-62 (determined spectrophotometrically) was found to be proportional to the amount of lytic activity measured, in agreement with previous reports (Hayashi *et al.*, 1965; Hartdegen and Rupley, 1967).

Optical rotatory dispersion measurements were made on a Cary Model 60 spectropolarimeter. The reduced mean residue rotation was calculated from the relation

$$[m]' = \frac{3}{n^2 + 2}\frac{M_0\alpha_{obsd}}{dC}$$

where $[m]'$ is the reduced mean residue rotation, n the index of refraction of the solvent, M_0 the mean residue molecular weight, α_{obsd} the observed rotation, C the concentration in g/100 ml, and d the path length in decimeters.

Tryptophan was estimated colorimetrically by reaction of the protein with 2-hydroxy-5-nitrobenzyl bromide in 8 M urea (pH 2.7) as described by Barman and Koshland (1967). The extent of the reaction was determined from the absorbance at 410 nm and the protein concentration was determined by amino acid analysis. In agreement with the results of Barman and Koshland (1967), somewhat lower values for native lysozyme were obtained when the disulfide bonds were not reduced and alkylated. Accordingly, the values for the oxidized proteins were corrected for this difference.

Results

Titration of Lysozyme with N-Methylnicotinamide Chloride in the Presence of Competitive Inhibitors. The titration of native chicken egg-white lysozyme with *N*-methylnicotinamide chloride yielded an apparently homogeneous complex displaying charge-transfer characteristics (Deranleau *et al.*, 1969). In order to establish whether *N*-methylnicotinamide was bound in the vicinity of he active-site "cleft" of the molecule, the titrations were repeated in the presence of two competitive inhibitors of the enzymatic function, *N*-AcGlu and tri-*N*-AcGlu.

The results of these experiments are shown in Figure 1. The titration data for the native enzyme are shown as a dashed line, and for clarity, the data points have been omitted for all experiments except those with 0.1 M *N*-AcGlu and 0.01 M tri-*N*-AcGlu. Within the limits of experimental error, the addition of *N*-acetylglucosamine prevented the formation of the nicotinamide complex in a competitive manner. The extinction coefficient (see legend to Figure 1) in the presence of *N*-AcGlu was essentially the same as observed for the native titration of all *N*-AcGlu concentrations studied, and a systematic decrease in the apparent binding constant of *N*-methylnicotinamide to lysozyme was observed with increasing concentrations of *N*-AcGlu. From these data, which are typical of competitive binding situations (see Appendix), the association constant of the *N*-AcGlu–lysozyme complex at each concentration was computed. The results are summarized in Table I and reflect a weak binding with regard to the inhibition of the formation of the *N*-methylnicotinamide complex.

The inhibition of the formation of the complex by tri-*N*-AcGlu was more striking, as shown by the data in Figure 1. As in the case of *N*-AcGlu inhibition, the extinction of the

[2] The twice-crystallized preparations of α-lactalbumin used as starting material in these experiments was generously supplied by Dr. Keith Brew, Department of Biochemistry, University of Leeds, Leeds, England.

[3] Abbreviations used are: *N*-AcGlu, *N*-acetylglucosamine; tri-*N*-AcGlu, *N*-acetylglucosamine trimer; NBS, *N*-bromosuccinimide; HNB, 2-hydroxy-5-nitrobenzyl bromide.

[4] The authors are indebted to Dr. Philip E. Wilcox for the use of the Cary Model 15.

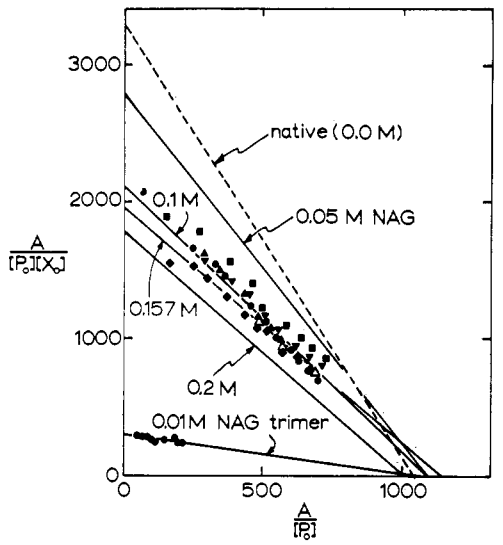

FIGURE 1: Linear (Scatchard type) plot of the titration curves of lysozyme with *N*-methylnicotinamide chloride in the presence of various competitive inhibitors. *A* is the absorbance of the complex at 350 nm, P_0 is the total protein concentration in moles per liter, and X_0 is the total *N*-methylnicotinamide chloride concentration in moles per liter. The association constant, k, is the negative of the slope and the extinction coefficient, ϵ, is the intercept of the A/P_0 axis. The dashed line is for the native titration (Deranleau *et al.*, 1969).

complex was unchanged but the apparent binding constant was appreciably lowered, being equal to 0.3 l. mole^{-1} as compared with 3.2 for the native complex. The inhibition constant, representing the binding of tri-*N*-AcGlu, was calculated to be 970 l. mole^{-1}. These results clearly establish that known competitive inhibitors of the enzymatic function also compete with *N*-methylnicotinamide.

In view of these observations, it was of interest to test whether *N*-methylnicotinamide chloride acted as a competitive inhibitor of cell lysis by the enzyme. Assays were performed as described previously (Bradshaw *et al.*, 1967) except that the 0.1% NaCl (w/v) was replaced by 1 M *N*-methylnicotinamide. Control experiments were carried out with 1 M NaCl. Under these conditions, the lytic activity in both the *N*-methylnicotinamide and control experiments was reduced to about 2% of that observed under normal conditions. Although no significant difference was observed with *N*-methylnicotinamide in the assay medium as compared with NaCl solutions of equivalent ionic strength, the results were not accurate enough to establish whether *N*-methylnicotinamide acts as a competitive inhibitor for cell wall lysis. However, lysozyme solutions preincubated with *N*-methylnicotinamide are fully active upon 1 to 300 dilution of the *N*-methylnicotinamide, indicating the probable reversible nature of the complex.

Titrations of Oxidized Lysozyme Derivatives with N-Methylnicotinamide Chloride. Several investigations (Hayashi *et al.*, 1965; Blake, 1967; Hartdegen and Rupley, 1967) have demonstrated specific destruction of individual tryptophanyl residues in lysozyme by various oxidative means. Trp-108 can be selectively destroyed by iodine oxidation and Trp-62 by reaction with NBS. In both cases, the final product appears to be oxindole. Since the *N*-AcGlu inhibition studies suggested that a tryptophan in the active site was a likely binding site for *N*-methylnicotinamide, derivatives of lysozyme, prepared by reaction with submolar quantities of NBS to avoid overoxidation, were tested for their ability to form a complex with a charge-transfer absorption.

Two derivatives were prepared, and their properties are summarized in Table II. Within experimental error, the extent of modification of Trp-62 was found to be equal to the per cent lytic activity destroyed, in agreement with the observations of Hartdegen and Rupley (1967) and Hayashi *et al.* (1965). The optical rotatory dispersion curves obtained

TABLE I: Binding Data for Inhibition of *N*-Methylnicotinamide–Lysozyme Complex by *N*-Acetylglucosamine and *N*-Acetylglucosamine Trimer.

Inhibitor Concn (No. of Expt)	n[a]	$\bar{k}_{app}$[b]	$\bar{\epsilon}$[c]	$\bar{k}_Y$[d]
None (7)	60	3.2 ± 0.2[e]	1040 ± 40	
N-Acetylglucosamine				
0.05 M (3)	30	2.56 ± 0.24	1100 ± 60	5.0 ± 7
0.1 M (6)	50	1.92 ± 0.25	1090 ± 76	6.7 ± 2.4
0.157 M (2)	16	1.75 ± 0.15	1130 ± 32	5.3 ± 1.2
0.2 M (2)	15	1.76 ± 0.01	995 ± 10	4.1 ± 0.6
		n-weighted mean[f]	1090 ± 60	5.7 ± 3.2
N-Acetylglucosamine trimer				
0.01 M (1)	9	0.30	982	970

[a] Number of data points. [b] Mean apparent association constant plus and/or minus standard deviation (liter per mole, observed slope of Scatchard plot). See Appendix. [c] Mean extinction coefficient at 350 nm. [d] Mean association (inhibition) constant for *N*-AcGlu binding plus and/or minus standard error (liter per mole). See Appendix, eq 7a and 9a. [e] $\bar{k}_X$. [f] $\Sigma_i n_i \bar{k}_i / \Sigma_i n_i$.

TABLE II: Properties of Lysozyme Derivatives Prepared by Oxidation with *N*-Bromosuccinimide.

Derivative	% Act.[a]	Trp Content[b]	$-[m]'_{233}$ (deg)
Native lysozyme	100	6.00	−4723
Ox lysozyme 1	35	5.25	−4704
Ox lysozyme 2	17	5.12	−4694

[a] Values given are per cent relative to native lysozyme assayed under identical conditions (Bradshaw *et al.*, 1967). [b] Determined spectrophotometrically by the method of Barman and Koshland (1967).

TABLE III: Values Assigned to the Binding Constant (k) and the Extinction Coefficient (ϵ) for *N*-Methylnicotinamide–Lysozyme and –α-Lactalbumin Complexes.

	Lysozyme		α-Lactalbumin
	Native	Oxidized	Native
k_1	3.2	3.2	
ϵ_1	1040	1040	
k_2	0	0.8	0.8
ϵ_2	0	700	650

were superimposable and the $[m]'_{233}$ value for each sample was identical. These results suggest that a limited modification of lysozyme has been carried out and that this modification has not disrupted the gross three-dimensional structure of the enzyme despite the loss in lytic activity.

The titration data for the complex between the oxidized samples and *N*-methylnicotinamide chloride are shown in Figure 2. As in Figure 1, the titration of the native enzyme is shown as a dashed line. In each oxidized lysozyme sample, the extent of complexation with *N*-methylnicotinamide was markedly reduced but, in addition, a distinct curvature in each plot was observed. The titration data for native lysozyme (Deranleau *et al.*, 1969) are consistent with a model in which a single site (or a single set of identical sites) is present, characterized by the constants: k = 3.2 l. mole^{-1} and ϵ 1040 l. mole^{-1} cm^{-1} (Table III, column 1). The data for the oxidized samples, which contain some native molecules and some modified molecules, cannot be adequately described by a multiple equilibrium model involving one site on the native and two sites on the oxidized lysozyme. However, the data fit a model in which there are two different sites, one each on the native and oxidized samples, and are also consistent with the assignment of the amount of remaining native enzyme to the per cent lytic activity in both preparations of oxidized lysozyme. Determination of the parameters k_1,ϵ_1 and k_2,ϵ_2 for the two sites (see Appendix) was made on a trial and error basis to generate the best fit to the observed data. The solid lines shown in Figure 2 were obtained using the constants listed in the second column of Table III, with α, the fraction of lytic activity remaining (see Appendix), equal to 0.35 for ox-lysozyme 1, and 0.17 for ox lysozyme 2 (Table II).

The third set of data points shown in Figure 2 describe the titration of bovine milk α-lactalbumin with *N*-methylnicotinamide chloride. Only a very weak complex was noted, compared with lysozyme. However, as with native lysozyme, a linear plot was obtained, suggesting the presence of only a single class of sites. The fitted values for k and ϵ for a single-site model of the *N*-methylnicotinamide–α-lactalbumin complex are given in Table III.

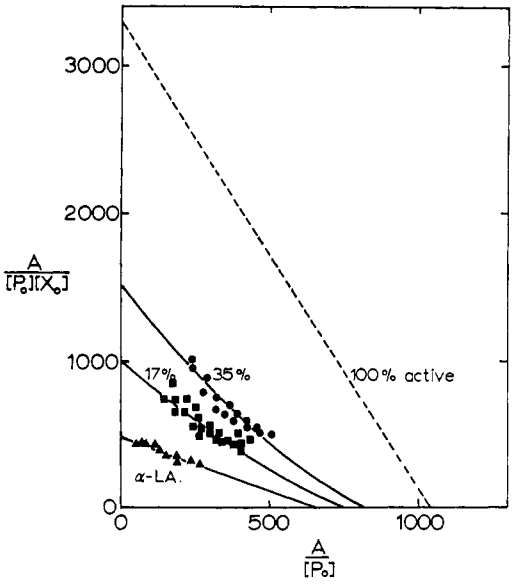

FIGURE 2: Linear (Scatchard type) plot of the titration curves of various oxidized lysozyme samples and bovine α-lactalbumin with *N*-methylnicotinamide chloride. A description of the axis is given in Figure 1. The dashed line represents the titration of native lysozyme (Deranleau *et al.*, 1969) and (●) and (■) are for the titrations of lysozyme oxidized with *N*-bromosuccinimide to the extent that they contained 35 and 17% lytic activity, respectively. The titration of α-lactalbumin is indicated by (▲). The solid lines are theoretical curves, see Appendix (eq 5a and 6a) for details.

Discussion

The competition experiments with *N*-AcGlu and tri-*N*-AcGlu indicate that the tryptophan residue which serves as the primary binding site for *N*-methylnicotinamide (Deranleau *et al.*, 1969) is a part of the inhibitor binding site. According to the crystallographic evidence (Blake *et al.*, 1967b), this observation implicates the indole moieties of Trp-62, and/or -108 as possible binding sites. The indole rings of Trp-63, and to an even greater extent of Trp-108, are clearly more "buried" in the crystallographic structure than that of Trp-62, which appears to be the only indole site available for a flat, face-to-face interaction with *N*-methylnicotinamide. Such a flat, face-to-face geometry—or one closely approximating it—is specifically implied for the *N*-methylnicotinamide–lysozyme complex on the basis of the observed extinction coefficients and shape of the charge-transfer band (Deranleau *et al.*, 1969) in comparison with model studies (Deranleau and Schwyzer, 1970; Deranleau *et al.*, 1970).

The observation that tri-*N*-AcGlu is more effective than *N*-AcGlu as a competitor of *N*-methylnicotinamide binding might be expected on the basis of the crystallographic data (Blake *et al.*, 1967b). Although both *N*-AcGlu and tri-*N*-AcGlu are (presumably) hydrogen bonded to the indole nitrogens of Trp-62 and -63, tri-*N*-AcGlu effectively overlaps the entire ring face of Trp-62 whereas *N*-AcGlu does not. On steric grounds, tri-*N*-AcGlu would thus be the better inhibitor. From the standpoint of the relative equilibrium constants of the two derivatives, the present results are in qualitative agreement with studies on the inhibition of the catalytic function of lysozyme by *N*-AcGlu and tri-*N*-AcGlu (Rupley *et al.*, 1967). However, the inhibition constants determined by competition with *N*-methylnicotinamide are lower than those found by others: approximately 6 l./mole for *N*-AcGlu binding as compared with 15–65 l./mole (depending on the method used) found by others (Dahlquist

et al., 1966; Rupley *et al.*, 1967; Dahlquist and Raftery, 1968). The source of this discrepancy is unknown, and since the errors in the determination of the binding constants for *N*-AcGlu and tri-*N*-AcGlu by the present method are large (involving as they do a subtraction between two small and relatively imprecise numbers), it is questionable whether further effort in this direction is warranted. It is possible, of course, that a procedural error was involved—*e.g.*, control of pH (the solutions were buffered by lysozyme itself)—or that the competitive inhibition model chosen to fit the data was in fact inappropriate. With respect to the latter, however, it was the simplest of several models tried which gave more or less self-consistent results with the four concentrations of *N*-AcGlu employed in this study. Again, the lack of accuracy in the data does not invite further examination of alternate interaction mechanisms. In any case, the data qualitatively support the tentative conclusion that *N*-methylnicotinamide binds in the active center of lysozyme, probably to the indole ring of Trp-62.

The oxidation studies bear out the hypothesis that the indole moiety is the primary *N*-methylnicotinamide binding site. Oxidation with *N*-bromosuccinimide resulted in a disappearance of the primary site, although a second, weaker site became available at the same time. Hayashi *et al.* (1965) have shown that Trp-62 is the residue which reacts with *N*-bromosuccinimide. The most likely product is oxindole, and the change from trigonal to tetrahedral hybridization at the C-3 position of the indole ring should result in a perturbation of the conformation of the entire side chain (Blake, 1967). It is therefore possible that, on oxidation of Trp-62, a local conformational change takes place in which a second indole ring becomes available for interaction with *N*-methylnicotinamide.[5] By inference, this would be the indole ring of Trp-63, the closest tryptophan both in space and presumably in sensitivity to conformational change at the Trp-62 residue.

While the inference of Trp-63 as a secondary site for *N*-methylnicotinamide binding is conjectural, it is of considerable interest that α-lactalbumin, in which Trp-62 is replaced by Ile-59 (Brew *et al.*, 1967), also binds *N*-methylnicotinamide with an association constant and extinction coefficient closely resembling oxidized lysozyme. If indeed the same site is utilized in both cases (Trp-63 in lysozyme, Trp-60 in α-lactalbumin), this is evidence for the three-dimensional homology of the two proteins, as proposed by Hill *et al.* (1968) on the basis of sequence homology.

Acknowledgment

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[5] Oxindole does not show a charge-transfer interaction with *N*-methylnicotinamide; D. A. Deranleau and R. A. Bradshaw, unpublished observations.

Appendix

Binding by n Independent Species, Each with One Site (Oxidation Studies). Let $P_{o,1}, P_{o,2}, \ldots, P_{o,n}$ be the total (constant) concentrations of the various species of P, each of which has a single site for combination with an adsorbate molecule. If X is the (variable) concentration of the added adsorbate, and C_i is the concentration of the complex between X and the ith species of P, $C_i = s_i P_{o,i}$, where $s_i = k_i X/(1 + k_i X)$ is the *saturation fraction* of the ith species of P ($0 \leqslant s_i \leqslant 1$), and k_i is the formation constant. When the adsorbate is in considerable excess over all the $P_{o,i}$, its total concentration, X_0, can be used as an approximation to the free concentration $X = X_0 - \Sigma_i C_i$ (Deranleau, 1969). For a Beer–Lambert law dependence of the absorbance A on the concentration of complexes, the observation equation for the entire system is

$$A = \sum_i A_i = \sum_i \epsilon_i C_i = \sum_i \epsilon_i s_i P_{o,i} \tag{1a}$$

which has a maximum $A_{max} = \Sigma_i A_{max,i} = \Sigma_i \epsilon_i P_{o,i}$ as $X \to \infty$. The operationally defined saturation fraction of the entire system is thus an average weighted according to the absorptivity coefficients, ϵ_i, and the total concentrations of the constant (dilute) species: $\bar{s} = A/A_{max} = \Sigma_i \epsilon_i s_i P_{o,i}/\Sigma_i \epsilon_i P_{o,i}$, $0 \leqslant \bar{s} \leqslant 1$, and differs from the stoichiometric saturation fraction $\bar{\bar{s}} = \Sigma_i C_i / \Sigma_i P_{o,i} = \Sigma_i s_i P_{o,i}/\Sigma_i P_{o,i}$, $0 \leqslant \bar{\bar{s}} \leqslant 1$, by the constants ϵ_i.

For purposes of comparison, it will be convenient to derive the form of the theoretical curves corresponding to this model in terms of a plot of A/P_oX *vs.* A/P_o (Scatchard, 1949; Deranleau, 1969), where $P_o = \Sigma_i P_{o,i}$ is the total concentration of all species of P. Differentiating A and A/X (eq 1a) with respect to X, the slope of the plot at a given X is

$$-\frac{\dfrac{\partial(A/X)}{\partial X}}{\dfrac{\partial A}{\partial X}} \equiv -\frac{\dfrac{\partial(/AP_oX)}{\partial X}}{\dfrac{\partial(A/P_o)}{\partial X}} = \frac{\sum_i \epsilon_i k_i^2 P_{o,i}(1 + k_i X)^{-2}}{\sum_i \epsilon_i k_i P_{o,i}(1 + k_i X)^{-2}} = (\bar{k})_X \tag{2a}$$

The slope of the plot thus varies continuously with the addition of X, and the apparent formation constant $(\bar{k})_X$ (tangent to the curve at a given X), is weighted according to the ϵ_i and $P_{o,i}$, as expected. For the limiting slopes at both ends of the plot ($\bar{s} = 0$ and $\bar{s} = 1$, respectively), we have

$$\begin{aligned} \lim_{X\to 0} (\bar{k})_X &= \sum_i \epsilon_i k_i^2 P_{o,i} \Big/ \sum_i \epsilon_i k_i P_{o,i} = (\bar{k})_0 \\ \lim_{X\to \infty} (\bar{k})_X &= \sum_i \epsilon_i P_{o,i} \Big/ \sum_i \epsilon_i k_i^{-1} P_{o,i} = (\bar{k})_\infty \end{aligned} \tag{3a}$$

and the intercepts, from eq 1a, are both weighted according

$$\begin{aligned} \lim_{X\to 0} A/P_oX &= \sum_i \epsilon_i k_i P_{o,i} \Big/ \sum_i P_{o,i} = (\bar{\epsilon k})_0 \\ \lim_{X\to \infty} A/P_o &= \sum_i \epsilon_i P_{o,i} \Big/ \sum_i P_{o,i} = (\bar{\epsilon})_\infty \end{aligned} \tag{4a}$$

to the $P_{o,i}$. We note that the weighting factors for the apparent

formation constant ($\bar{k}$) change during the course of the experiment (eq 3a), from a type of Z average to a reciprocal average.

For the particular model under consideration in this work, the $P_{o,i}$, two in number (native or "active," and oxidized or "inactivated"), are related by the expressions

$$\alpha = P_{o,1}/P_o,\ 1 - \alpha = P_{o,2}/P_o;\ P_{o,1} + P_{o,2} = P_o \quad (5a)$$

where α is the fraction of molecules with the original binding site remaining after oxidation with NBS. We have defined α operationally as the fraction of enzymatic activity remaining after oxidation. The relevant equations for the calculation of the theoretical binding curves are then

$$A/P_o = \alpha\epsilon_1 s_1 + (1 - \alpha)\epsilon_2 s_2$$

$$\lim_{X\to 0} A/P_oX = \alpha\epsilon_1 k_1 + (1 - \alpha)\epsilon_2 k_2 \quad (6a)$$

$$\lim_{X\to \infty} A/P_o = \alpha\epsilon_1 + (1 - \alpha)\epsilon_2$$

where $s_1 = k_1X/(1 + k_1X)$ and $s_2 = k_2X/(1 + k_2X)$

Two Adsorbate Species Competing for the Same Site (N-AcGlu Titrations). The equations for this model are well known, and the derivation will not be repeated here. If only the complex with adsorbate X gives rise to an absorbance $A = \epsilon_X C_X$, where C_X is the concentration of the complex with X, then a plot of A/P_oX *vs.* A/P_o will be a straight line with slope (apparent association constant)

$$-a_1 = k_X/(1 + k_Y Y_o) = -k_{app} \quad (7a)$$

Here k_Y is the formation constant for the binding of adsorbate Y at (constant) total concentration Y_o, and k_X is the formation constant for the binding of adsorbate X (variable concentration). The intercepts of the plot are

$$\lim_{X\to 0} A/P_oX = \epsilon_X k_X/(1 + k_Y Y_o)$$

$$\lim_{X\to \infty} A/P_o = \epsilon_X \quad (8a)$$

Ignoring a possible error in Y_o, the error Δk_Y in k_Y for errors Δk_{app} and Δk_X in the slope in the presence and absence, respectively, of inhibitor Y, is given by

$$\Delta k_Y{}^2 = \left(\frac{\partial k_Y}{\partial k_{app}}\right)^2 \Delta k_{app}{}^2 + \left(\frac{\partial k_Y}{\partial k_X}\right)^2 \Delta k_X{}^2$$
$$= k_{app}{}^{-2} Y_o{}^{-2}(k_X{}^2 k_{app}{}^{-2}\Delta k_{app}{}^2 + \Delta k_X{}^2) \quad (9a)$$

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