Binding of N-Methyl Nicotinamide Chloride by Tryptophan Residues of α-Lactalbumin

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SUMMARY

Studies of the binding of N-methyl nicotinamide chloride by α-lactalbumin show that complex formation occurs with 1 of 4 tryptophan residues at pH 6 whereas two form complexes at pH 2 as a result of acid denaturation. Alkaline denaturation likewise leads to an increase in exposure of tryptophans, and three of the four groups form complexes at pH 11. From a consideration of previous studies and a recently proposed molecular model for α-lactalbumin, the residues involved in binding N-methyl nicotinamide chloride have been tentatively identified as tryptophan-118, tryptophan-104, and tryptophan-60.

Previous studies with α-lactalbumin have shown changes in the environment of tryptophanyl side chains as a result of acid and alkaline conformational changes which occur with this protein (1-7). Studies utilizing acid and alkaline difference spectra (1, 5) have shown changes in the neighborhood of one or more of the tryptophanyl side chains relative to the native protein. Similar results have been obtained by fluorescence spectroscopy (3) and circular dichroism measurements (6). Solvent perturbation difference spectral measurements at acid pH indicate, however, that the changes observed during the acid transition do not involve a net change in exposure to solvent of tryptophanyl side chains relative to the native protein. Interference due to tyrosyl ionization precludes the use of the latter technique at alkaline pH.

Since the solvent perturbation method does not unambiguously differentiate between partial exposure of many groups or complete exposure of a few groups (8), we have investigated the use of N-methyl nicotinamide chloride for titrating exposed tryptophanyl side chains (9-11). This reagent forms a 1:1 charge-transfer complex with tryptophanyl residues, and because of the planar interaction of the two ring systems needed for complex formation, requires that the tryptophanyl group be exposed at the protein surface and essentially unhindered. N-Methyl nicotinamide chloride has been reported to form a charge-transfer complex with tryptophanyl residues (9-11). This reagent forms a 1:1 charge-transfer complex with tryptophanyl residues (9-11). This reagent forms a 1:1 charge-transfer complex with tryptophanyl residues (9-11). This reagent forms a 1:1 charge-transfer complex with tryptophanyl residues (9-11).

This paper concerns itself with the use of N-methyl nicotinamide chloride as a reagent for titrating the exposed tryptophanyl side chains of α-lactalbumin, and illustrates the potential of this technique when used in conjunction with solvent perturbation and chemical methods for mapping the topography of proteins.

A tentative identification of the binding sites for N-methyl nicotinamide chloride has been made from the molecular model of α-lactalbumin proposed by Browne et al. (13).

EXPERIMENTAL PROCEDURE

Materials—N-Methyl nicotinamide chloride was obtained from Sigma Chemical Co. (lot No. 59B-5310), ε = 0.01 molar cm⁻¹ at 350 nm. N-Acetyl tryptophan ethyl ester was obtained from Mann Research Laboratories, Inc. Lysozyme was a salt-free preparation obtained from Worthington Biochemical Co., lot LY 618. α-Lactalbumin was prepared according to the method of Robbina and Kronman (14).

Binding Studies—For the binding experiments protein concentrations were 5 mg per ml in aqueous solution. Protein solutions were adjusted to the appropriate pH by the addition of 0.1 M HCl or 0.1 M NaOH and measured on a Radiometer Instrument, model PH 4. Solid N-methyl nicotinamide chloride was added to the protein solutions; the amount added was determined by weighing the cuvettes before and after each addition, and concentrations were corrected for volume changes occurring (10). Spectra were measured at room temperature on a Cary 15 recording spectrophotometer. Eight to ten spectra were obtained for each protein solution at a variety of molar excesses.

Protein concentrations were measured at 280 nm with ε₂₈₀ = 20.1 (15) and ε₂₈₀ = 26.5 (16) for α-lactalbumin and lysozyme, respectively. The assumed molecular weight for α-lactalbumin was 14,500, and for lysozyme, 14,400.

RESULTS

N-Acetyl Tryptophan Ethyl Ester and Lysozyme—Preliminary to binding studies with α-lactalbumin, we investigated the pH dependency of complex formation between N-acetyl tryptophan ethyl ester and N-methyl nicotinamide chloride at pH 2, 6, and 11. We also repeated the binding studies of Deradeau et al. (10) with lysozyme at pH 5.5. The value of the association constant, K, and the molar extinction coefficient, ε, for the complex were obtained from the slope and intercept of a plot of A/P₀X₀ versus A/P₀ according to the relationship (10)

\[ \frac{A}{P₀X₀} = K \left( \frac{ε - \frac{A}{P₀}}{P₀} \right) \]

where A = absorbance due to complex at 350 nm; P₀ = total protein concentration in moles per liter; and X₀ = total nicotinamide chloride concentration in moles per liter.
TABLE I

| Constant | α-Lactalbumin | Lysozyme | N-Acetyl tryptophan ethyl ester |
|----------|---------------|----------|--------------------------------|
|          | pH 2         | pH 6     | pH 11                          |
| $K$ (M$^{-1}$) | 4.9 ± 0.8  | 3.8 ± 0.5 | 6.6 ± 0.7                      |
| $\epsilon$ | 2277 ± 34    | 1240 ± 11 | 3141 ± 31                      |
| $N^b$    | 2.2           | 1.2      | 3.3                            |
|          | pH 5.5       | pH 2     | pH 6                           |
| $K$ (M$^{-1}$) | 3.7 ± 0.3, 3.2$^a$ | 1230 ± 7 | 1040$^a$                       |
| $\epsilon$ | 825          | 815      | 810                            |

$^a$ Reference 10.

$^b$ Moles of tryptophan residues formed in a complex per mole of protein = $\epsilon/1135$; 1135 is the average molar extinction coefficient for the lysozyme complex from this study and Reference 10 and is the value assumed for the complex formation of a single tryptophan residue. See text for details.

The data for N-acetyl tryptophan ethyl ester when plotted according to Equation 1 gave a straight line at all pH values. The values of the constants $K$ and $\epsilon$ (Table I) show little or no pH dependence of the molar extinction coefficient; however, the association constant is significantly higher at high pH. The values of the constants at pH 6 are also in good agreement with those found for N-acetyl tryptophan and N-acetyl tryptophanamide in water (9).

The lysozyme data were linear when plotted according to Equation 1 (Fig. 1). The value of $K$ was 3.7 M$^{-1}$ and $\epsilon$ was 1230 M$^{-1}$ cm$^{-1}$. Those findings are in reasonable agreement with those of Demanleu et al. (10) (Table I).

Bradshaw and Demanleu (11) have demonstrated that only 1 mole of nicotinamide is bound to lysozyme at tryptophan-82. Therefore, the molar extinction coefficient of the tryptophan-nicotinamide complex is significantly higher when present in lysozyme as compared to model tryptophan compounds. There are no data available to judge whether this is a general rule with proteins or whether it is a reflection of the environment of the complex in lysozyme. With α-lactalbumin, however, as shown below, the binding data are most reasonably interpreted if the number of binding sites are estimated with the molar extinction coefficient determined for lysozyme rather than those from model compounds.

Native α-Lactalbumin—Titration of α-lactalbumin at pH 6 with N-methyl nicotinamide chloride results in formation of a yellow-colored complex due to charge-transfer complex formation with tryptophan (9-11) (Fig. 2). Complex formation was instantaneous and was monitored by measuring the increase in absorbance at 350 nm upon addition of N-methyl nicotinamide chloride, as described by Demanleu et al. (10, 11).

The titration data plotted according to Equation 1 (Fig. 1) give a linear plot indicating a single class of binding sites in the native protein. The regression line was determined by the method of least squares, and the values of the constants estimated

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Fig. 1. Plot of N-methyl nicotinamide chloride binding to lysozyme at pH 5.5. α—α-Lactalbumin at pH 6, ○—○. Protein concentration, 3.5 × 10$^{-4}$ M.

Fig. 2. Absorption spectra showing charge-transfer complex formation between N-methyl nicotinamide chloride and α-lactalbumin. Protein concentration, 3.5 × 10$^{-4}$ M; N-methyl nicotinamide chloride concentration, 0.5 μM. a, α-lactalbumin at pH 6; b, 1:20 dilution of a; c, α-lactalbumin plus N-methyl nicotinamide chloride at pH 6; d, α-lactalbumin plus N-methyl nicotinamide chloride at pH 2; e, α-lactalbumin plus N-methyl nicotinamide chloride at pH 11.
Although we cannot offer a firm explanation for this anomalous behavior, some recent observations made in our laboratory indicate that it may arise from the well known tendency of \(\alpha\)-lactalbumin to associate near pH 6 (15). This latter behavior appears to be related to the method of purification of the protein. 

\section*{DISCUSSION}

The present study indicates that native \(\alpha\)-lactalbumin has only one tryptophanyl group sufficiently exposed at the surface so that the planar geometry of the indole and pyridinium rings required for charge-transfer complex formation (9) can occur. By contrast solvent perturbation measurements show that an average of two tryptophanyl groups are exposed in the native protein (2). Chemical reactivity studies with \(N\)-bromosuccinimide (16) and spectrophotometric titrations with \(N\)-methyl nicotinamide (17) indicate that the protein has two sites of high affinity for N-methyl nicotinamide while Guire (17) reported that only one tryptophanyl group is exposed at the surface of the protein. The significantly higher association constants of the alkaline-denatured protein compared to the native and acid-denatured proteins also indicate that the conformation in the vicinity of the tryptophanyl side chains of the alkaline-denatured proteins facilitate binding of \(N\)-methyl nicotinamide. These observations are in general agreement with previous studies of the state of the tryptophanyl residues in \(\alpha\)-lactalbumin.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{binding.png}
\caption{Plot of \(N\)-methyl nicotinamide chloride binding to \(\alpha\)-lactalbumin. Protein concentration, 3.5 \times 10^{-4} \text{M}; \circlearrowright, pH 2; \circlearrowleft, pH 11.}
\end{figure}
with the model of α-lactalbumin proposed by Browne et al. (13) on the basis of its homology with hen lysozyme.

The present study shows that the conformational changes occurring at acid pH with α-lactalbumin result in complete exposure of an additional tryptophanyl side chain for complex formation with N-methyl nicotinamide. Solvent perturbation also shows an average of two exposed groups, and suggests that, at pH 6, tryptophanyl residues 104 and 118 are most probably the groups perturbed. These observations agree with circular dichroism results (6) which indicate that one or more tryptophanyl side chains are in a less constrained environment relative to the native protein as a result of the acid transition and are therefore capable of forming a complex with N-methyl nicotinamide. Chemical modification studies at acid pH with 2-hydroxy-5-nitrobenzyl bromide (20) show that tryptophan-118 and tryptophan-104 are reactive; however, tryptophan-26, which is thought to be completely buried, is unexpectedly reactive (see below). Tryptophan-60 shows very little reactivity. As pointed out by Barman (20), the relatively high concentration of acetone (10%) used in the reaction mixture may induce additional conformational changes which could lead to further exposure of groups, and thus account for the unexpected reactivity of tryptophan-26.

At alkaline pH three tryptophanyl side chains form a complex with N-methyl nicotinamide, showing their exposure at the protein surface. This is in keeping with the results of circular dichroism measurements which show that the tryptophanyl side chains of the alkalin-denatured protein are less constrained at high pH (6) than at pH 0 and that the environment in the vicinity of a disulfide bridge of α-lactalbumin is changed at high pH (see below).

Although no definitive information is available at present on the tertiary structure of α-lactalbumin it will be useful to consider the environment of the tryptophanyl side chains as seen in the model of α-lactalbumin proposed by Browne et al. (13) and based on the similarity in amino acid sequence of bovine α-lactalbumin and hen lysozyme, and to compare it with the observations of the present study and previous work. The description of the model is taken from the work of Kronman et al. (7) and is presented below.

α-Lactalbumin contains four tryptophanyl side chains, and the model indicates that only one of them, tryptophan-26, can be considered as being completely buried in the interior of the protein. Tryptophan-118 lies exposed on the surface with little apparent hindrance from other side chains. Tryptophan-104 and tryptophan-60 appear to be less exposed. These residues lie in a cleft-like region of the molecule, homologous with the active site of lysozyme. Tryptophan-104 is somewhat hindered by tyrosine-103 and by other side chains in the cleft. Tryptophan-60 appears to be less accessible than tryptophan 104, and is surrounded by several hydrophobic residues in addition to tyrosine-103. The proximity of disulfide bridge 73-92 might also shield tryptophan-60 from contact with solvent.

On the basis of the chemical reactivity studies described previously and the environment of the tryptophanyl residues seen in the α-lactalbumin model, it is most likely that tryptophan-118 is the exposed group forming a complex at pH 6 in the native protein. Similar reasoning suggests that tryptophan-104 is most likely the other group perturbed by solvent in the native protein, and that this group is subsequently available for complex formation at acid and alkaline pH as a result of the conformational changes which occur under these conditions. The previously described observations from circular dichroism studies at high pH, as well as the environment surrounding tryptophan-60, as seen in the molecular model, make it likely that this residue is involved in binding at alkaline pH along with tryptophan-118 and tryptophan 104.

The observations of this study when interpreted in conjunction with previous work on α-lactalbumin provide support for the proposed molecular model of this protein. However, a direct verification of the sites involved in binding will require the preparation and titration of suitably modified derivatives.

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