Chemical Characterization of the Dapsone Binding Site of Lysozyme[1]

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Dapsone (4,4′-diaminodiphenyl sulfone, DDS) is one of the most popular drugs used for the treatment of leprosy. Although it has been in use for more than three decades, its mode of action, bioavailability, and interactions at the molecular level are still not clear. Earlier work regarding its binding to serum albumin ([1, 15, 16, 21]) was carried out using the equilibrium dialysis technique. Spectroscopic techniques, however, provide more specific information on the nature of the binding phenomenon. In this respect, fluorescence spectroscopy provides direct information regarding the involvement of the aromatic residues of proteins in the association process. Infrared (IR) and laser Raman spectroscopy can be employed to examine the vibrational modes of either the drug or the protein interlocked in the complex formation, and nuclear magnetic resonance (NMR) studies can give much detailed information on the involvement of protons of specific amino acid residues in the interaction. Work using these techniques has already been initiated ([4]). The possible mode of interaction of the drug at the membrane level also has been recently reported ([5,6]).

We report here the involvement of tryptophan and lysine residues of hen egg white lysozyme at the dapsone binding site, using fluorescence spectroscopy. In addition to their basic importance, these studies could be clinically relevant because lysozyme levels have been shown to increase in some leprosy cases, especially in lepromatous leprosy ([19, 20]), and if lysozyme associates with dapsone, it could reduce the availability of free dapsone in the blood and thus limit drug efficiency.

MATERIALS AND METHODS

Chemicals. Hen egg white lysozyme was from Sigma Chemical Company, St. Louis, Missouri, U.S.A., and methanol was purchased from E. Merck, Darmstadt, West Germany. All of the buffers were stored at 4°C. Acetate buffer (pH 4.5) and phosphate buffers (pH 6.2 and 8.0) with ionic strengths ranging from 0.05 M to 0.2 M were used.

Fluorescence spectroscopy. All fluorescence readings were taken on an Aminco Bowmann spectrophotometer using a wide-range photomultiplier tube and an X-Y recorder. A quartz cell, 1 cm path length with 4 ml capacity, was used for this purpose. Oxygen was not excluded from the reaction mixture. The excitation wavelength used was 280 nm.

The stock solutions of dapsone (1 mg/ml) and protein (10 mg/ml) were made in methanol and distilled water, respectively. An aliquot of protein (0.5 ml) was made up to 10 ml with the required buffer. The experimental solutions were prepared by mixing the required aliquot (10–400 μl) of stock dapsone solution with 0.5 ml of protein and diluting to 10 ml with the required buffer. Since dapsone also has some absorption at 280 nm, correction for the inner filter effect was applied using the method of Velick, *et al.* ([23]).

Assay of lysozyme. Lysozyme activity was measured by monitoring the rate of change in turbidity of *Micrococcus luteus* cell suspension ([8]). Lyophilized cells (1 mg/ml) were suspended in phosphate buffer (pH 6.2, 0.06 M). The buffer (2.7 ml) and the above substrate suspension (0.3 ml) were taken in a glass cuvette to which 10 μl of enzyme (10 μg/10 μl) was added and stirred well. The turbidity at 420 nm was measured at 15-sec intervals using a Hitachi spectropho-

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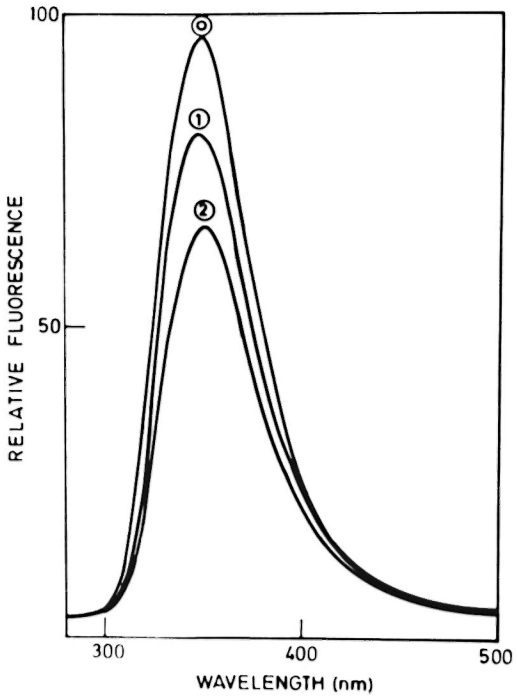

FIG. 1. The decrease in fluorescence yield at 380 nm with increasing concentrations of dapsone.

◎: [lysozyme] = 6.6×10^{-6} M
①: [dapsone]:[lysozyme] = 1
②: [dapsone]:[lysozyme] = 2

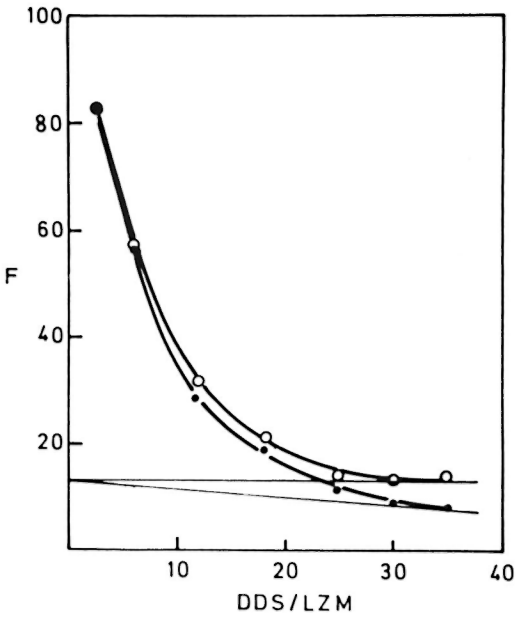

FIG. 2. Correction for inner filter effect. Corrected curve (-o-o-); fluorescence quenching observed (-●-●-).

tometer. The effect of dapsone was measured by using the above buffer containing 0.01–0.1 M dapsone. The rate constant, k, was calculated from these data.

Modification of lysozyme residues. Tryptophan (Trp) 62 was oxidized with N-bromosuccinimide (NBS) to oxindole using the method of Hayashi, *et al.* ([9]), and Trp-108 was oxidized by iodine according to the method of Imoto, *et al.* ([11]). Both of the modified forms of the protein were purified on a chitin-coated cellulose column ([13]). The NBS-treated enzyme had 50% of the original activity, as also shown by Spande and Witkop ([22]), while the iodine-oxidized enzyme had only 2% of the original enzyme activity. Both had retained their spectral characteristics although their fluorescence and absorbance at 280 nm was less than that of the native enzyme. Acetylation with acetic anhydride was carried out according to the method of Neuberger and Davies ([3]). The extent of acetylation was determined by the Moore and Stein method ([18]). Acetylation was complete to the extent of 95%. The acetylated enzyme was inactive toward *Micrococcus luteus* in 0.06 M phosphate buffer. It was homogenous on disc electrophoresis ([14]).

RESULTS AND DISCUSSION

The fluorescence of hen egg white lysozyme, an enzyme, was quenched by dapsone. The decrease in the fluorescence yield at 350 nm with increasing concentration of drug is presented in Figure 1. There was no shift in the wavelength with quenching. The correction for the inner filter effect is indicated in Figure 2. The open circles show the actual fluorescence after correction for the absorption of dapsone. These corrected values were used in all further calculations. The quenching was independent of pH and ionic strength, showing that ionic forces were not participating in the binding. The quenching of the fluorescence can be due to collisional quenching or due to the formation of a static complex ([7]). Which of these two mechanisms dominate can be determined by the nature of a Stern-Volmer graph. A linear Stern-Volmer graph was obtained at lower ratios of drug to protein (Fig. 3), whereas an upward-curving graph was seen at higher

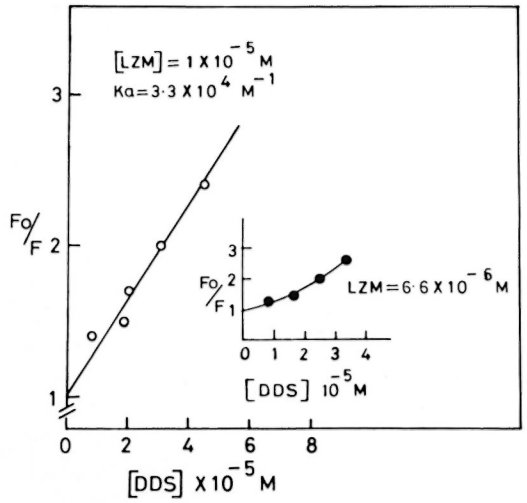

FIG. 3. Stern-Volmer plot for the association of dapsone with lysozyme. Inset = Stern-Volmer plot for higher drug:protein ratios.

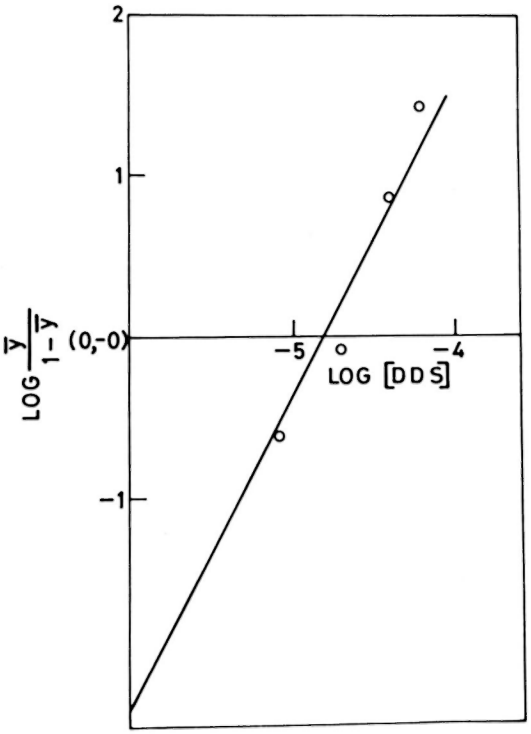

FIG. 4. Hill plot for the association of dapsone with lysozyme. The data from Fig. 3 inset were used in this figure.

drug-to-protein ratios (insert, Fig. 3). The straight line graph is shown to indicate formation of ([8]) a 1:1 complex. The Ka calculated from this graph was $3.3 \times 10^4\ M^{-1}$, whereas the upward-curving graph at higher concentrations confirms the formation of a static complex. However, the Scatchard plot obtained from the data in the inset was upward sloping. Such plots are indicative of cooperative phenomena ([17]). In order to check this possibility, a Hill plot ([2]) was drawn. Figure 4 shows the linear Hill plot obtained with the above data. The slope, $n = 2$, yields the number of binding sites; $n > 1$ indicates an interaction between sites, thus confirming the operation of cooperative forces in the drug protein complex. The average Ka from the Hill plot for both sites was $6.6 \times 10^4\ M^{-1}$, showing that both sites have similar Ka = $3.3 \times 10^4\ M^{-1}$. These results clearly show that dapsone binds hen egg white lysozyme, and that at higher concentrations the binding is cooperative in nature.

The intrinsic fluorescence of lysozyme measured at 340 nm is due to its tryptophan residues ([12]). Hence, any changes observed in the fluorescence of proteins are known to be due to the changes in the environment of the tryptophan residues. Dapsone also quenched the fluorescence of free tryptophan (Fig. 5), indicating that free tryptophan also associated with the antileprosy drug with a Ka = $6.7 \times 10^4\ M^{-1}$. This gives further support to the possibility of tryptophan participating in the binding process. The fact that dapsone showed greater affinity for the free amino acid as compared to the native lysozyme suggested that some other amino acid residues prevent dapsone binding to the protein. This was evident when it was found that modification of the lysine residues increased the affinity of the drug to the protein. Thus, the presence of lysine residues causes the difference in the binding of the drug to free tryptophan and the binding of the drug to the tryptophan moiety of the protein. The results, therefore, suggest involvement of tryptophan in the dapsone–lysozyme complex.

Although lysozyme has six tryptophan residues, not all contribute equally toward the fluorescence of the protein. In fact, Trp-108 and Trp-62 have been shown to be the major contributors toward the fluorescence of this protein ([12]). Out of the six residues, only Trp-108 and Trp-62 can be selectively modified. Modification of any other residue

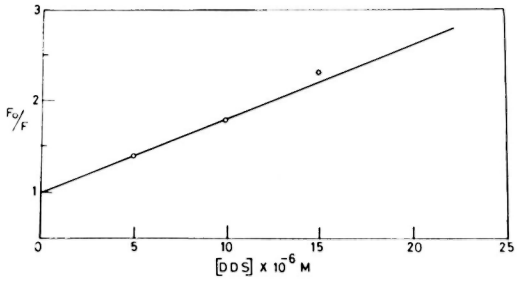

FIG. 5. Stern-Volmer plot for the association of free tryptophan with dapsone; [tryptophan] = 1×10^{-5} M.

results in oxidation of some of the other tryptophan residues also. Hence, to enable us to determine the role of the two fluorescing tryptophan residues in the interaction of lysozyme with dapsone, Trp-108 and Trp-62 were specifically modified. Modification of either brought about a decrease of 40% in the affinity of the drug to the protein (The Table), showing that both of these residues play a role in the drug binding.

Modification of lysine residues caused an increase in binding by 40% (The Table). Therefore the positive charges contributed by the ϵ-amino group of lysine seem to repel the drug. This shows that the side-chain residues on the surface of the protein exercise discrimination in allowing the association of the ligands with the protein.

The activity of the enzyme was not affected by dapsone in the concentration range of 0.01–0.1 M. Serum from leprosy patients was also incubated with dapsone (4–40 μg/0.1 ml of serum) for 7 days and 17 days at 0°C and 37°C. Neither time nor temperature caused any change in the activity of serum lysozyme, indicating that dapsone binds outside the active site region with this enzyme.

Similar experiments with serum albumin have shown that dapsone also quenches the intrinsic fluorescence of both bovine and human serum albumin. Earlier workers ([1, 15, 16, 21]) have also shown by other techniques that dapsone binds the serum albumins. Acetylation of the lysine residues of albumin also was found to increase the affinity of the drug for the protein, just as was observed for lysozyme.

Dapsone is a hydrophobic, aromatic molecule and probably interacts with hydrophobic regions of the protein. Since modification of tryptophan residues decreases the binding substantially, the drug most probably stacks over it through π–π interactions, forming a dark complex. These conclusions are supported by the observations of Helene and Lancelot ([10]) who have shown stacking of other aromatic molecules, such as purine and pyridine bases with tryptophan, using NMR spectroscopy.

THE TABLE. *Association constants of dapsone with modified lysozyme.*

Lysozyme	Ka (M^{-1})
Native	6.0×10^4
Trp-62 modified	2.5×10^4
Trp-108 modified	2.2×10^4
Lysine modified	1.0×10^5

Lysozyme has been shown to be increased in the serum of leprosy patients, particularly those with lepromatous leprosy. Since dapsone binds to lysozyme, this could lead to a decrease in the concentration of free dapsone in the blood compared with healthy individuals. Acetylation of the protein increases the affinity of the drug for the protein, suggesting that, at least theoretically, dapsone should not be administered along with aspirin which is known to acetylate albumin. Hence, if administered with aspirin, dapsone will bind more to the protein and this could, at least theoretically, reduce the availability of free drug and, therefore, tend to decrease its efficacy.

SUMMARY

The binding of dapsone to hen egg white lysozyme has been studied using fluorescence spectroscopy. At low concentrations the drug binds lysozyme with a Ka = 3.3×10^4 M^{-1} forming a 1:1 complex. At high concentrations the protein was found to bind the drug in a cooperative manner at two sites with an average association constant of 6.3×10^4 M^{-1}. Both Trp-108 and Trp-62 of lysozyme are involved in the association process. Acetylation of the lysine residues increased the affinity of the drug to the protein. However, drug association showed no effect on the enzymatic activity of the protein.

RESUMEN

Se estudió el enlazamiento de la dapsona a la lisozima de la clara de huevo usando la espectroscopía de fluoresencia. A concentraciones bajas, la droga se unió a la lisozima con una Ka = 3.3×10^4 M^{-1}, formando un complejo 1:1. A concentraciones altas, la proteína

fijó a la droga en dos sitios y de manera cooperativa con una Ka = 6.3 × 10^4 M^{-1}. Tanto el triptofano (Tri) 108 como el Tri 62 de la lisozima estuvieron involucrados en el proceso de enlazamiento. La acetilación de los residuos de lisina aumentó la afinidad de la droga a la proteína. Sin embargo, la asociación de la droga no mostró ningún efecto sobre la actividad enzimática de la proteína.

RÉSUMÉ

Le couplage de la dapsone au lysosyme de blanc d'oeuf de poule a été étudié au moyen de la spectroscopie en fluorescence. A faibles concentrations, le médicament se copule au lysosyme, avec un Ka = 3,3 × 10^4 M^{-1}, formant un 1:1 complex. A hautes concentrations, on a observé que la protéine se couplait aux médicaments d'une façon coopérative, à deux endroits, avec une constante moyenne d'association de 6,3 × 10^4 M^{-1}. Tant la Trp-108 que la Trp-62 du lysosyme était impliquée dans le processus d'association. L'acétylation des résidus de la lysine augmentait l'affinité du médicament pour la protéine. L'association médicamenteuse n'a cependant montré aucun effet sur l'activité enzymatique de la protéine.

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