Involvement of Histidine and Tryptophan Residues of Glutamine Binding Protein in the Interaction with Membrane-Bound Components of the Glutamine Transport System of *Escherichia coli*†

Arthur G. Hunt‡ and Jen-shiang Hong*

ABSTRACT: We treated the glutamine binding protein with diethyl pyrocarbonate (DEPC) and *N*-bromosuccinimide (NBS) to modify respectively the sole histidine and tryptophan residues and examined the effect of these modifications on the ability of the binding protein to bind glutamine as well as the ability to restore glutamine transport in membrane vesicles of *Escherichia coli*. Under the conditions used, both DEPC and NBS markedly inhibited the ability to restore glutamine transport in vesicles without any significant effect on glutamine binding. Moreover, saturating quantities of glutamine had no protective effect on the inactivation of the binding protein by DEPC or NBS. Fluorometric measurement and amino acid analysis indicate that the inactivation of the binding protein in restoring vesicle transport by NBS can be attributed to the oxidation of a single tryptophan residue. Similar analysis and the inability of hydroxylamine to reverse the effect of DEPC indicate that the effects of DEPC can probably be attributed to alterations of the sole histidine and/or one or more lysine residues of the binding protein. We conclude that the glutamine binding protein possesses at least two largely nonoverlapping functional domains, one responsible for glutamine binding and the other for the interaction with the other components of the glutamine transport system.

Several transport systems in *Escherichia coli* require, in addition to one or more membrane-bound components, periplasmic substrate binding proteins for their activity (Wilson, 1978). These proteins are generally small, possess no enzymatic activity, and bind their respective substrates with very high affinity. The binding properties of several of these proteins have been studied in some detail [reviewed in Wilson & Smith (1978)]. However, information concerning the interaction between these proteins and the membrane-bound components of their respective transport systems has been sorely lacking, largely because of the lack of a system suitable for the study of these interactions.

Among the best characterized of these binding proteins is the glutamine binding protein. This protein, a small, basic polypeptide of molecular weight 26 000, binds glutamine with a K_D of 3×10^{-7} M, a value similar to the K_m for glutamine of the transport system in whole cells (Weiner & Heppel, 1971). This binding is both rapid and reversible. Futhermore, this binding is relatively insensitive to extremes of temperature, pH, and ionic strength (Hunt & Hong, 1983). Detailed genetic (Masters & Hong, 1981a) and biochemical (Masters & Hong, 1981b) studies have demonstrated that this protein is required for the activity of the osmotic shock sensitive glutamine transport system in *E. coli*. However, very little is known about the interaction of this protein with the other components of the glutamine transport system.

Recently, we described the successful restoration of glutamine transport in isolated *E. coli* membrane vesicles by the glutamine binding protein (Hunt & Hong, 1981). Here, we use this system to begin a study of the interaction of the glutamine binding protein with isolated membrane vesicles. Specifically, we demonstrate that treatment of the glutamine binding protein with diethyl pyrocarbonate (DEPC)[1] or *N*-bromosuccinimide (NBS) abolishes the ability of the glutamine binding protein to restore transport in isolated membrane vesicles but does not affect the glutamine binding properties of this protein.

Materials and Methods

Materials. [^{3}H]Glutamine was obtained from New England Nuclear. DEPC was obtained from Sigma; the container was stored immersed in desiccant at 4 °C. NBS and hydroxylamine hydrochloride were from Eastman. NBS was recrystallized from glacial acetic acid. Nitrocellulose filters used for binding protein assays were from Schleicher & Schuell, as were cellulose acetate filters used for transport assays. All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Growth Media. Two derivatives of *E. coli* K12 were used in this study; binding protein was prepared from PSM2, a strain that overproduces this protein and presumably the other components of theglutamine transport system (Masters & Hong, 1981a); vesicles were prepared from PSM116, a derivative of PSM2 containing a point mutation in the structural gene for the glutamine binding protein (Masters & Hong, 1981a,b).

Bacteria were grown in minimal salts medium (Vogel & Bonner, 1956) with 0.5% carbon source. Methionine was added to 400 μM and thiamin to 40 μM.

Purification of the Glutamine Binding Protein. The glutamine binding protein was purified from succinate-grown PSM2 essentially as described previously (Hunt & Hong, 1981; Masters & Hong, 1981b), except the CM-cellulose-concentrated shock fluid was concentrated by ultrafiltration through an Amicon membrane (type PM10) at 50 psi, instead of by ammonium sulfate precipitation.

Preparation of Isolated Membrane Vesicles. Membrane vesicles, containing 0.1 mM NAD, 0.1 mM ADP, and 10 μM CoA, were prepared from glycerol-grown PSM116 as de-

† From the Department of Cell Physiology, Boston Biomedical Research Institute, Boston, Massachusetts 02114. *Received August 13, 1982.* This work was supported by Grants GM22576 and GM29853 from the National Institute of General Medical Sciences.

‡ Present address: Laboratory of Plant Molecular Biology, The Rockefeller University, New York, NY 10021.

[1] Abbreviations: DEPC, diethyl pyrocarbonate; NBS, *N*-bromosuccinimide; CM, carboxymethyl; CoA, coenzyme A; BSA, bovine serum albumin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/83/0422-0851$01.50/0 © 1983 American Chemical Society

scribed previously (Hunt & Hong, 1981).

Glutamine Transport Assays. Glutamine transport in isolated membrane vesicles was assayed at a saturating concentration of glutamine (36.4 μM) as described (Hunt & Hong, 1981), except that the final external pH of the vesicles plus binding protein suspension was adjusted to 6.0 with 1 M KH_2PO_4 prior to transport. Transport was assayed at 37 °C.

Glutamine Binding Assays. Glutamine binding to the glutamine binding protein in the presence of a saturating glutamine concentration (13.4 μM) was routinely assayed by a filter assay previously described (Hunt & Hong, 1981). Equilibrium dialysis was used for the determination of the binding constants of DEPC- and NBS-treated binding protein. A 250-μL sample of binding protein (0.02–0.2 mg/mL in 2 mg/mL BSA) was dialyzed against 10 mL of [^{3}H]glutamine (49.9 mCi/mmol, 0.18–1.82 μM) in a carbon-free salts medium (N^+C^-) (Gutnick et al., 1969) containing a subsaturating quantity of chloroform, for 16 h at 37 °C, in a shaking water bath. The glutamine concentrations of the external medium and the binding protein solution were determined by liquid scintillation counting.

Treatment of Glutamine Binding Protein with DEPC. Glutamine binding protein (1 mg/mL) in 30 mM KP_i, pH 6.0, was incubated for varying lengths of time (10–90 min) with different concentrations of DEPC (0.1–5 mM) at 37 °C. DEPC was added as 0.02 volume in absolute ethanol. Reactions were stopped by putting the reaction mixture (50 μL) onto a Sephadex G-25 column (~300 μL total volume) and eluting the binding protein with 300 μL of 10 mM Tris-HCl, pH 7.0. This binding protein was used directly for transport assays.

Alternatively, larger volumes of binding protein were reacted with DEPC, the pH was adjusted to 7.0 with 1 M K_2HPO_4, and the mixtures were dialyzed overnight against 1000 volumes of 10 mM Tris-HCl, pH 7.0.

Difference Spectra of DEPC-Treated Glutamine Binding Protein. Difference spectra were obtained by dividing binding protein solutions of 0.1–0.4 mg/mL into two aliquots in different cells and adding DEPC (in absolute ethanol) to 5 mM to the sample cell; difference spectra were recorded every 10 min until no further changes could be seen by using a Perkin-Elmer Model 557 double-beam spectrophotometer. These reactions were carried out at room temperature (24 °C). Once the reactions were completed, the modified binding protein was dialyzed against 500 volumes of 10 mM Tris-HCl, pH 7.0, for 16 h at 4 °C, and assayed for binding and transport.

Hydroxylamine Treatment of DEPC-Inactivated Binding Protein. Hydroxylamine hydrochloride (2 M), neutralized to pH 7.0 with KOH, was added to aliquots of DEPC-treated binding protein, obtained as described above, to a final concentration of 0.4 M. After 90 min at 37 °C, the solution was dialyzed against 5000 volumes of 10 mM Tris-HCl, pH 7.0, and 0.1 mM acetyl phosphate (eliminate excess hydroxylamine) for 16 h at 4 °C. This treatment has no effect on the binding and transport properties of native glutamine binding protein.[2] Transport and binding were assayed as described above.

Treatment of Glutamine Binding Protein with N-Bromosuccinimide. Glutamine binding protein was treated with *N*-bromosuccinimide (Spande & Witkop, 1967) in 0.1 M sodium acetate, pH 4.5, for 5 min at 37 °C. Samples were used directly for transport assays except when glutamine was used. In this case, the protein was passed through a Sephadex G-25 column equilibrated with 10 mM Tris-HCl, pH 7.0, before transport assays.

[2] A. G. Hunt and J. Hong, unpublished experiments.

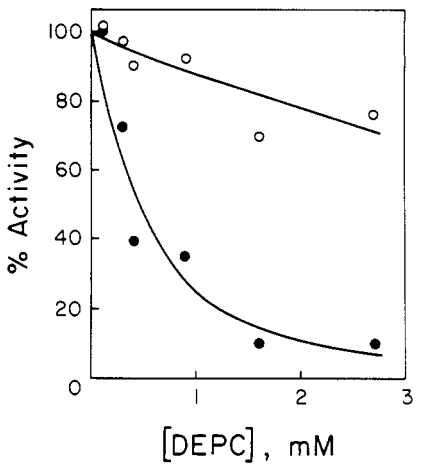

FIGURE 1: Effect of DEPC on the binding and transport properties of the glutamine binding protein. Glutamine binding protein (50 μg) was reacted with varying concentrations of DEPC for 90 min at 37 °C and recovered as described under Materials and Methods. Glutamine binding (○) and the ability of binding protein to restore transport in vesicles (●) were then assayed as described under Materials and Methods. Blank values, obtained by assaying binding and transport in the absence of binding protein, were subtracted from the experimental values and the data normalized with respect to untreated binding protein.

Amino Acid Analysis. Native and modified glutamine binding proteins were hydrolyzed in 6 N HCl for 24 h at 105 °C in evacuated, sealed tubes. The amino acid analysis was performed on a Beckman-Spinco amino acid analyzer.

Protein Determinations. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Results

Effect of DEPC Treatment on the Transport and Binding Properties of the Glutamine Binding Protein. Treatment of the glutamine binding protein with varying concentrations of DEPC for 90 min at 37 °C changed the transport properties of this protein dramatically. Under these conditions, the ability of this protein to restore glutamine transport in vesicles was inhibited by 50% with 0.5 mM DEPC and by 90% with 1.6 mM DEPC (Figure 1). The ability of the binding protein to bind glutamine, on the other hand, was relatively unaffected by such treatments (Figure 1). Binding protein that had been inactivated by treatment with 5 mM DEPC for 80 min at 37 °C bound glutamine with a K_D of 2.9×10^{-7} M, a value essentially identical with that of the native binding protein (Weiner & Heppel, 1971). These results demonstrate that DEPC treatment affects an amino acid residue (or residues) involved in the interaction of liganded binding protein with membrane-bound components of the glutamine transport system but has no effect on those parts of the molecule involved in the binding of glutamine.

Saturating quantities of glutamine (70 μM) had no effect on the inactivation of the binding protein by DEPC (data not shown). The absence of any protective effect of glutamine suggests that the amino acid residue (or residues) altered by DEPC is not located in the binding site of this protein but located elsewhere in the molecule.

Effect of NBS Treatment on the Transport and Binding Properties of the Glutamine Binding Protein. Like DEPC treatment, NBS treatment of glutamine binding protein selectively affected the transport properties of this protein. As shown in Figure 2, oxidation of 0.4 tryptophan residue (20%

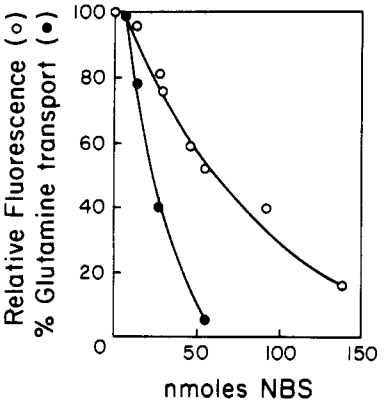

FIGURE 2: Effect of NBS on transport properties of the glutamine binding protein. Glutamine binding protein (0.5 mg/mL) was reacted in 0.1 M sodium acetate, pH 4.5, with varying concentrations of NBS for 5 min at 37 °C. Oxidation of tryptophan (○) was monitored by fluorometry with excitation at 280 nm and emission at 340 nm with a Perkin-Elmer fluorometer. Glutamine transport (●) in isolated membrane vesicles was assayed as described under Materials and Methods by using approximately 12 μg of binding protein.

of residues), measured by the fluorescence at 340 nm, inhibited the ability of the protein to restore glutamine transport in vesicles by 60%, and oxidation of approximately one tryptophan residue essentially completely abolished its ability to restore transport. However, binding protein that has one of its two tryptophan residues oxidized retained the full ability to bind glutamine with a K_D of 2.9×10^{-7} M. Moreover, a saturating concentration of glutamine (1.6 mM) was without effect on this inactivation by NBS (data not shown). These results are very similar to those obtained with DEPC and indicate that NBS affects the transport-restoring ability of glutamine binding protein by altering one of the two tryptophan residues located outside of the glutamine binding site of this protein.

Identity of the Amino Acid Residues Altered by DEPC. Under the conditions described here, DEPC selectively reacts with histidine residues in proteins and polypeptides (Miles, 1977). This reaction is characterized by the appearance of an absorption maximum between 230 and 250 nm. Furthermore, the product of the reaction of DEPC with histidine, *N*-(carboxyethyl)histidine, can react with hydroxylamine to regenerate histidine, thereby reversing any effect DEPC may have on a protein's function. DEPC-inactivated glutamine binding protein did indeed show an absorption maximum at 239 nm (Figure 3), indicating a reaction with the sole histidine residue of the glutamine binding protein. However, prolonged hydroxylamine treatment of DEPC-inactivated binding protein did not restore any transport-stimulating activity to this protein. DEPC has also been reported to react with tyrosine, lysine, and serine residues in proteins and polypeptides (Miles, 1977). Amino acid analysis of DEPC-inactivated glutamine binding protein indicated that the six tyrosine and six serine residues of this protein were not affected by this treatment (data not shown). Because of the large number of lysine residues in this protein [27; see Weiner & Heppel (1971)], we could not rule out the possibility that one or more of these residues was affected by DEPC using amino acid analysis. The two tryptophan residues of this protein were also unaffected by DEPC treatment, since the tryptophan fluorescence in DEPC-inactivated binding protein was identical with that of untreated binding protein (data not shown).

Amino Acid Analysis of Glutamine Binding Protein Treated with NBS. NBS is known to oxidize tryptophan, tyrosine, and histidine residues in proteins and polypeptides (Spande & Witkop, 1967). Furthermore, NBS has been shown to oxidize the two tryptophan residues of the glutamine binding protein, in a reaction that was prevented by saturating concentrations of glutamine (Weiner & Heppel, 1971). The NBS inactivation described above was not affected by the presence of glutamine. Under the conditions used, the oxidation of one of the two tryptophan residues was sufficient to inactivate the ability of the binding protein to restore transport. Furthermore, amino acid analysis indicated that the histidine and tyrosine residues of the binding protein were not affected by such a treatment (data not shown). These observations indicate that inactivation of transport activity by NBS is solely due to oxidation of one of two tryptophan residues of the glutamine binding protein.

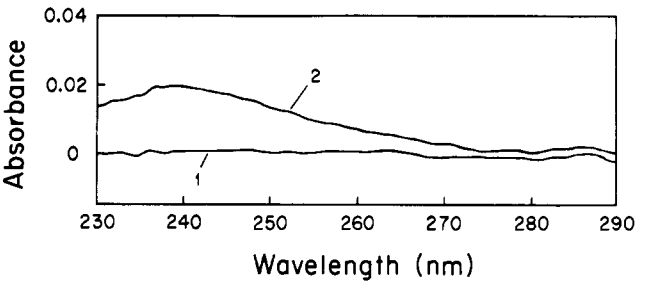

FIGURE 3: Difference spectrum of DEPC-treated glutamine binding protein. Glutamine binding protein (260 μg/mL, in 10 mM KP_i, pH 6.0) was reacted for 90 min at room temperature with 5 mM DEPC. The difference spectrum between this sample and an identical, untreated sample was then measured with a Perkin-Elmer model spectrophotometer. Glutamine binding protein so treated was inactivated by greater than 90% (data not shown). Difference spectrum at time = 0 min (1) and time = 90 min (2).

Discussion

The periplasmic glutamine binding protein from *E. coli* has two distinctive properties that are related to its function: it binds glutamine, selectively and with high affinity (Weiner & Heppel, 1971); and it restores the ability to transport glutamine to spheroplasts and isolated membrane vesicles (Hunt & Hong, 1981; Masters & Hong, 1981b). The results presented in this paper demonstrate that the ability of the glutamine binding protein to restore transport in membrane vesicles can be altered without affecting the glutamine binding properties of this protein.

Apparently, more than one amino acid residue of the glutamine binding protein is involved in the effects described in this paper. Modification of one of the two tryptophan residues by NBS is sufficient to destroy the ability of the binding protein to restore transport in vesicles yet does not affect the glutamine binding properties of this protein. DEPC treatment also causes these effects, but apparently by affecting a different amino acid residue, or residues. Under conditions used here, DEPC reacts with the sole histidine residue of the glutamine binding protein. The inability of hydroxylamine to reverse the inactivation caused by DEPC suggests that another residue, or residues, may also be altered by this reagent and that it is this residue that is involved in the effects seen here. Besides histidine, DEPC has been shown to react with tyrosine, serine, and lysine residues in proteins (Miles, 1977). The absence of any change of absorbance near 280 nm argues against the possibility that a tyrosine residue is involved in the action of DEPC. Amino acid analysis indicated that the tyrosine and serine residues of the glutamine binding protein were not affected by DEPC treatment. Moreover, fluorometric experiments showed that DEPC did not affect the tryptophan residues of this protein. However, our results cannot rule out a possible reaction of DEPC with any of the 27 lysine residues

of this protein. Presently, the effects of DEPC described here can be best attributed to alterations of the sole histidine and/or one or more lysine residue of the glutamine binding protein.

From the results described here, it can be concluded that the glutamine binding protein possesses at least two largely nonoverlapping functional domains. One of these is responsible for the binding of glutamine and is not affected by the treatments used in these experiments. The other is involved in the interaction of the binding protein with the other components of the glutamine transport system and contains the residues altered by DEPC and NBS.

The participation of periplasmic binding proteins in active transport via so-called shock-sensitive transport systems has been well-established, primarily through genetic means, and in at least two cases biochemically as well. The binding properties of several of these proteins have been studied in much detail (Wilson & Smith, 1978). On the other hand, studies of the interaction of these proteins with membrane-bound components of their respective transport systems have been few. Ames & Spudich (1976) have presented genetic studies indicating a direct interaction between the histidine binding protein of *Salmonella typhimurium* and the membrane-bound product of the *his*P gene. Newcomer et al. (1981) have investigated conformational changes induced by ligand binding by the arabinose binding protein of *E. coli* by using X-ray crystallography and have pinpointed those parts of the molecule that change upon ligand binding. These parts of the protein have been suggested to be prime candidates for the sites of interaction of the arabinose binding protein with membrane-bound components of the high-affinity arabinose transport system. In this paper, we have used chemical modification with DEPC and NBS to separate the binding and transport domains of the glutamine binding protein and have implicated at least two, and possibly more, of the amino acid residues involved in the interaction of binding protein with the membrane-bound components of the glutamine transport system. These studies were made possible by an isolated membrane vesicle system capable of binding protein dependent glutamine transport (Hunt & Hong, 1981). Further studies utilizing this system, and involving different chemical modifications, localized and directed mutagenesis of glutamine binding protein, and various biophysical tools should greatly enhance our understanding of binding protein dependent glutamine transport in *E. coli*.

Acknowledgments

We thank Dr. Rene C. Lu for performing the amino acid analysis for us.

Registry No. Diethyl pyrocarbonate, 1609-47-8; *N*-bromosuccinimide, 128-08-5; L-glutamine, 56-85-9; L-tryptophan, 73-22-3; L-lysine, 56-87-1; L-histidine, 71-00-1.

References

Ames, G. F.-L., & Spudich, E. N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 5447–5451.

Gutnick, D., Calvo, J. M., Klopotowski, T., & Ames, B. N. (1969) *J. Bacteriol. 100*, 215–219.

Hunt, A. G., & Hong, J. (1981) *J. Biol. Chem. 256*, 11988–11991.

Hunt, A. G., & Hong, J. (1983) *Biochemistry* (preceding paper in this issue).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Masters, P. S., & Hong, J. (1981a) *J. Bacteriol. 147*, 805–819.

Masters, P. S., & Hong, J. (1981b) *Biochemistry 20*, 4900–4904.

Miles, E. W. (1977) *Methods Enzymol. 47*, 431–442.

Newcomer, M. E., Lewis, B. A., & Quicho, F. A. (1981) *J. Biol. Chem. 256*, 13218–13222.

Spande, T. F., & Witkop, B. (1967) *Methods Enzymol. 11*, 498–522.

Vogel, H. R., & Bonner, D. M. (1956) *J. Biol. Chem. 218*, 97–106.

Weiner, J. H., & Heppel, L. A. (1971) *J. Biol. Chem. 246*, 6933–6944.

Wilson, D. B. (1978) *Annu. Rev. Biochem. 47*, 933–965.

Wilson, D. B., & Smith, J. B. (1978) in *Bacterial Transport* (Rosen, B. P., Ed.) pp 495–557, Marcel Dekker, New York.