Journal of Cellular Biochemistry 39:207–216 (1989)

Evidence for Two Distinct Conformations of the *Escherichia coli* Mannitol Permease That Are Important for Its Transport and Phosphorylation Functions

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Column chromatography of the *Escherichia coli* mannitol permease (mannitol-specific enzyme II of the phosphotransferase system) in the presence of deoxycholate has revealed that the active permease can exist in at least two association states with apparent molecular weights consistent with a monomer and a dimer. The monomeric conformation is favored by the presence of mannitol and by the phosphoenolpyruvate (PEP)-dependent phosphorylation of the protein. The dimer is stabilized by inorganic phosphate (Pi), which also stimulates phospho-exchange between mannitol and mannitol 1-phosphate (a partial reaction in the overall PEP-dependent phosphorylation of mannitol). Kinetic analysis of the phospho-exchange reaction revealed that Pi stimulates phospho-exchange by increasing the V_{max} of the reaction. A kinetic model for mannitol permease function is presented involving both conformations of the permease. The monomer (or a less-stable conformation of the dimer) is hypothesized to be involved in the initial mannitol-binding and PEP-dependent phosphorylation steps, while the stably associated dimer is suggested to participate in later steps involving direct phosphotransfer between the permease, mannitol and mannitol 1-phosphate.

Key words: sugar transport, bacterial phosphotransferase system, protein conformation, monomer-dimer equilibruim

Escherichia coli transports D-mannitol and a number of other sugars through its cytoplasmic membrane via a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) first identified by Kundig et al. [1]. The PTS carries out the concomitant transport and phosphorylation of these sugars resulting in sugar phos-

Abbreviations used: DTT, dithiothreitol; EI, enzyme I of PTS; HPr, heat-stable phosphocarrier protein of the PTS; PAGE, polyacrylamide gel electrophoresis; PEP, phosphoenolpyruvate; Pi, inorganic phosphate; PMSF, phenylmethylsulfonylfluoride; PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; SDS, sodiumdodecylsulfate.

Received July 8, 1988; accepted September 6, 1988.

phate inside the cell [2]. The overall process of transport and phosphorylation is shown in the following reaction sequence.

$$\text{PEP} + \text{HPr} \xrightarrow{\text{enzyme I}} \text{HPr-P} + \text{pyruvate} \quad (1)$$

$$\text{HPr-P} + \text{sugar (out)} \xrightarrow[\text{(enzyme III)}]{\text{enzyme II}} \text{HPr} + \text{sugar-P (in)} \quad (2)$$

Both enzyme I (EI) and HPr (a small heat-stable protein) are the nonspecific cytoplasmic proteins of the PTS, whereas enzymes II (EII's) are the integral membrane-bound proteins that are responsible for sugar-specific transport and phosphorylation. In addition to these components, some sugars, such as glucose, also require another soluble enzyme, enzyme III (EIII) which is phosphorylated by HPr-P. EIII then donates its phosphate to EII, which subsequently transports and phosphorylates the sugar across the membrane. Moreover, under appropriate conditions, EII's can also catalyze a transphosphorylation reaction as shown below:

$$[^{14}\text{C}]\ \text{sugar} + \text{sugar-P} \xrightarrow{\text{enzyme II}} [^{14}\text{C}]\ \text{sugar-P} + \text{sugar}$$

Because of the high activity of the mannitol permease (the mannitol-specific enzyme II, also called EII^{mtl}) in induced cells, and its stability in detergents, it has been studied extensively. SDS-polyacrylamide gel electrophoretic studies revealed that the purified permease comprises a single polypeptide chain with an approximate molecular mass of 60 ($\pm 5\%$) kDa [3], while a molecular mass of 68 kDa was deduced from the amino acid sequence derived from the nucleotide sequence of the mannitol permease gene (*mtlA*) [4]. The enzyme has also been extensively characterized [5–10].

Based upon the differences observed for the pH, temperature, and enzyme-concentration dependencies of the two phosphorylation reactions catalyzed by the mannitol permease, Saier [11] proposed that it exists in both monomeric and dimeric forms, and that the transphosphorylation reaction is catalyzed by the dimeric form of the enzyme. Subsequently, Roossien and Robillard [6] showed that a fraction of the phosphorylated form of the mannitol permease could be extracted from the membrane partially as a dimer under mild denaturing conditions. This was later confirmed by Stephan and Jacobson [7], who also showed that the unphosphorylated form of the enzyme could be extracted from the membrane partially as a dimer. Furthermore, it was demonstrated that conditions favoring the PEP-dependent reaction favored the monomeric form of the mannitol permease, while conditions stimulating the transphosphorylation reaction favored the dimer, at least as observed after mild SDS extraction of the membrane [7]. In contrast, recent experiments carried out by Robillard and Blaauw [9] and Pas et al. [10] suggested that the oligomeric (minimum dimer) form of the enzyme was necessary to catalyze both transphosphorylation and PEP-dependent phosphorylation reactions.

Thus, the experimental evidence to date suggests that the mannitol permease exists in at least two conformations, a monomer (or a dimer unstable to detergent extraction) and a stable dimer, both of which may be important for its function.

However, all the experiments in which these forms of the enzymes have been directly observed were carried out under denaturing conditions. In this report, we now provide direct evidence that these two forms of the mannitol permease occur under conditions that maintain the active structure of the enzyme. Under these conditions, it is shown that the monomer is the predominant form of the enzyme that has been phosphorylated by EI, HPr, and PEP and of the enzyme in the presence of the substrate mannitol (intermediates in the PEP-dependent reaction), while the dimer is stabilized by conditions favoring the transphosphorylation reaction. We also present a kinetic model for permease function, involving both conformations of the protein, that is consistent with these results as well as those of other workers.

MATERIALS AND METHODS

Materials

Sephacryl S-200 was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Sodium deoxycholate was purchased from Sigma (St. Louis, MO) and was recrystallized three times from acetone:water (5:1 v/v) prior to its use. [^{3}H]-mannitol and [^{14}C]-mannitol were purchased from New England Nuclear (Boston, MA). [^{125}I]-labeled *Staphylococcus aureus* protein A was purchased from ICN (Irvine, CA). All other chemicals and reagents used during this investigation were of analytical grade.

Preparation of Membrane Vesicles

Escherichia coli, strain KL141, was grown to midexponential phase in 2 liters of M-63 medium containing 0.5% mannitol under aerobic conditions at 37 °C [3]. Membrane vesicles were prepared by passage through a French pressure cell at 10,000 psi. The unbroken cells were removed by a low-speed centrifugation (3,000 *g*, 5 min), and the supernatant containing vesicles was centrifuged at 100,000 *g* for 2 h at 4°C. The pellet was resuspended in 30 mM Tris-HCl, pH 8.0, containing 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonylfluoride (PMSF) (to inhibit endogenous proteolysis).

Solubilization of Mannitol Permease From Membrane Vesicles

Membrane vesicles were extracted in the presence of 0.5% sodium deoxycholate for 30 min as described by Jacobson et al. [3]. The extracted membranes were centrifuged at 100,000 *g* for 2 h. The supernatant was stored at −70°C until further use.

Determination of Protein Concentration

Protein concentrations were determined by following the method of Bradford [12] using bovine serum albumin as a standard.

Measurement of Mannitol Permease Activity

Both PEP-dependent and transphosphorylation activities of the mannitol permease were determined as described before [3,5,7,8]. A cytoplasmic fraction from *Salmonella typhimurium*, strain LJ144 [3], was used as a source of HPr and enzyme I for PEP-dependent phosphorylation, after extensive centrifugation (100,000 *g* for 2 h, 3 times) to remove contaminating membrane vesicles.

Column Chromatography

A Sephacryl S-200 column (65 × 1.6 cm) was packed at 4°C and at a flow rate of 30 ml/h. The column was equilibrated with at least two column volumes of buffer (pH 8.4) containing 10 mM Tris-HCl, 0.1 M NaCl, 0.05 mM PMSF, 0.5 mM DTT, and 0.25% sodium deoxycholate (column buffer) at a flow rate of 15 ml/h. The column was standardized with molecular weight markers (Pharmacia Fine Chemicals). After standardization, 2.0 ml of extracted membrane supernatant (2.4 mg/ml total protein) was loaded and eluted with the column equilibration buffer. The fractions (1.5 ml) collected were assayed for mannitol permease activities. For some experiments, 0.1 M sodium phosphate or 40 mM mannitol was included in the column buffer (pH 8.4). In these cases, deoxycholate-extracted membrane supernatant (2.4 mg/ml protein) was incubated with 0.1 M sodium phosphate or 40 mM mannitol for 30 min at 20°C prior to chromatography. Fractions from chromatography in the presence of mannitol were dialyzed at 4°C against the column buffer without mannitol for 24 h prior to assay to remove the hexitol (which interfered in the radioassay). Chromatography was also conducted on the phosphorylated mannitol permease using a separately standardized column at room temperature but otherwise as described above. In this case, deoxycholate-extracted membrane supernatant (2.1 mg/ml protein) was incubated with saturating amounts of enzyme I and HPr of *Salmonella typhimurium* and 20 mM PEP for 30 min at 30°C prior to chromatography. As a control, a separate column was run under identical conditions but omitting PEP from the preincubation mixture.

Electrophoresis and Immunoblotting

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli [13]. Samples were prepared in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 2.5 mM sodium thioglycolate, and 0.003% bromphenol blue at 100°C for 5 min. The gels were stained with Coomassie brilliant blue or silver [14] depending upon the amount of protein loaded. Immunoblotting of the proteins separated by SDS-PAGE was performed following the method of Towbin et al. [15], with modifications as described by Stephan and Jacobson [8], using antiserum raised against purified mannitol permease [16].

RESULTS

Column Chromatography of the Mannitol Permease Under Various Conditions

Figure 1A shows the activity profile of the mannitol permease obtained following chromatography of a sodium deoxycholate extract of *E. coli* membranes on Sephacryl S-200 column in the presence of 0.25% deoxycholate. Two distinct peaks of activity corresponding in apparent size to monomer (65 kDa ± 5%) and dimer (130 kDa ± 10%) were obtained. These results were also confirmed (results not shown) by electrophoresis and immunoblotting of the separated fractions obtained following the chromatograpy (see Materials and Methods). Under these conditions, approximately twice as much mannitol permease activity eluted at a position corresponding to dimer, as eluted at the position corresponding to monomer (Table I).

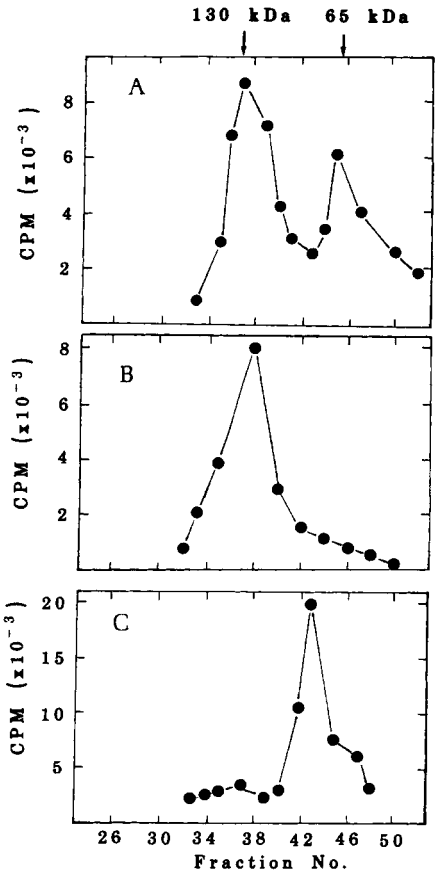

Fig. 1. Column chromatography of the mannitol permease in the presence of sodium deoxycholate (0.25%) under various conditions. **A:** No additions. **B:** +0.1 M sodium phosphate. **C:** +40 mM mannitol. The apparent molecular weights of the activity peaks (arrows) were determined by comparison with molecular weight standards run separately. 115 μl of each fraction was assayed for PEP-dependent phosphorylation of [^{14}C]- mannitol as described in Materials and Methods. Ordinate units are in CPM of [^{14}C]- mannitol 1-phosphate formed per 60 min. See Materials and Methods for further details.

TABLE I. Quantitative Effects of Inorganic Phosphate and Various Substrates on the Monomer-Dimer Equilibrium of the Mannitol Permease Observed by Sephacryl S-200 Column Chromatography.

Elution conditions[a]	% monomer[b]	% dimer[b]
Column buffer	39	61
+ 0.1 M sodium phosphate	10	90
+ 40 mM mannitol	89	11
+ EI + HPr	35	65
+ EI + HPr + 20 mM PEP	93	7

[a]For specific conditions refer to Materials and Methods.
[b]Relative to total monomer plus dimer; determined from the areas under the peaks of activity shown in Figures 1 and 2.

In contrast, if the chromatography was conducted in the presence of 0.1 M sodium phosphate, only a single major peak of mannitol permease activity was eluted at a position corresponding to that of a dimer (130 kDa; Fig. 1B; Table I). However, if the chromatography was conducted in the presence of mannitol, nearly all of the mannitol permease activity was eluted as monomer (Fig. 1C; Table I). Moreover, phosphorylation of the permease with EI, HPr, and PEP also led to predominantly monomer after chromatography (Fig. 2B; Table I) while incubation with EI and HPr alone had no significant effect on the elution profile as compared to elution of the permease in the absence of EI and HPr (cf. Figs. 1A, 2A; Table I).

Determination of Kinetic Parameters

Inorganic phosphate (Pi) has been shown both to stimulate the transphosphorylation reaction catalyzed by the mannitol permease and to increase coordinately the stability of permease dimer ([7] and this report). In order to investigate the mechanism of stimulation, we determined the kinetic parameters for the transphosphorylation reaction in the presence and absence of Pi. The results, summarized in Table II, show that Pi increases the V_{max} of this reaction 4–5- fold and increases K_m for both substrates approximately 2-fold. Thus, the conformation of the mannitol permease favoring the dimer appears to be more active than the monomer in the transphosphorylation reaction by virtue of a V_{max} effect rather than a K_m effect, at least in the presence of Pi. In contrast, Pi did not have any significant effect on the PEP-

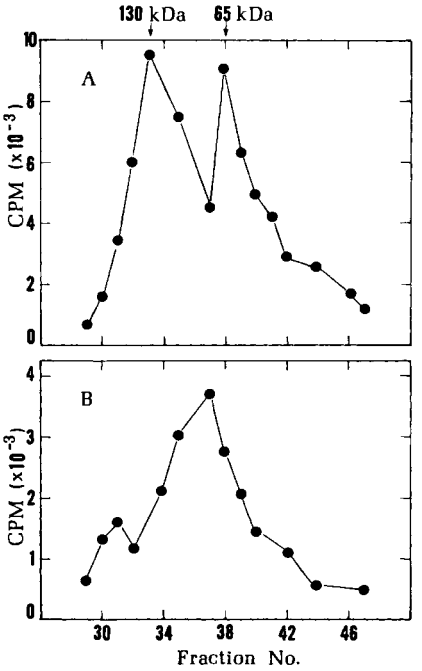

Fig. 2. Column chromatography of the mannitol permease in the presence of deoxycholate under nonphosphorylated **(A)** or phosphorylated **(B)** conditions. The elution positions of monomer (65 kDa) and dimer (130 kDa) are not directly comparable to those in Figure 1, since a separately standardized column was used for these experiments. See the legend to Figure 1 and Materials and Methods for details.

TABLE II. Kinetic Parameters for the Transphosphorylation Reaction in the Absence and Presence of Inorganic Phosphate*

	V_{max}		Apparent K_m	
Varied substrate	HEPES	Phosphate	HEPES	Phosphate
Mannitol 1-P[a]	3.19 (± 0.33)	15.30 (± 1.90)	0.124 mM (± 0.036)	0.290 mM (± 0.078)
[^{3}H]- Mannitol[b]	26.95 (± 2.12)	93.80 (± 15.96)	0.290 μM (± 0.028)	0.460 μM (± 0.057)

*Transphosphorylation assays were performed on membranes prepared from *E. coli*, strain KL141 [8] either in the presence of 0.1 M Na -HEPES or 0.1 M sodium phosphate, pH 7.0, as described in Materials and Methods.

[a]The concentration of mannitol 1-phosphate was varied from 0.01 to 0.1 mM. The concentration of [^{3}H]-mannitol was constant at 0.25 μM. Unit for V_{max} is pmol of [^{3}H]-mannitol 1-phosphate formed/30 min/mg protein.

[b]The concentration of [^{3}H]-mannitol was varied from 0.05 μM to 0.5 μM. The concentration of mannitol 1-phosphate was constant at 1 mM. Unit for V_{max} is pmol of [^{3}H]-mannitol 1-phosphate formed/30 min/mg protein.

dependent phosphorylation reaction with saturating EI and HPr under conditions in which the mannitol concentration was rate limiting (not shown).

DISCUSSION

The polypeptide chain (monomer) molecular weight of the *E. coli* mannitol permease in denaturing gels is 60 (± 5%) kDa [3] and 68 kDa from the deduced amino acid sequence [4]. Subsequently, Roossien and Robillard [6] using normal *E. coli* membranes, and Stephan and Jacobson [7], using membranes from minicells harboring a plasmid containing the *mtlA* gene, demonstrated that both a monomeric and a dimeric form of the permease can be extracted under mild denaturing conditions. However, the proportions of monomer and dimer extracted into SDS-containing buffer varied depending on the phosphorylation state of the permease, and on the presence or absence of mannitol or Pi [7]. The existence of a dimer of the purified permease was later confirmed by Roossien et al. [17] utilizing bifunctional sulfhydryl reagents. Most recently, using a radiation inactivation method, Pas et al. [10] obtained evidence suggesting that a dimer of the permease was necessary for both PEP-dependent and transphosphorylation reactions that was also consistent with kinetic studies of these reactions [9]. Thus, there is extensive evidence demonstrating that the mannitol permease exists in at least two different conformations which may be important for its various activities.

The present study was undertaken to determine whether subunit interactions of the permease could be studied conveniently under nondenaturing conditions, and if so, whether the effects of substrates, effectors, and phosphorylation on these interactions were the same for the active enzyme as had previously been shown for the partially denatured permease [7]. Detergents such as sodium cholate, sodium deoxycholate, or octylglucoside, because of their high critical micelle concentration, can be used to determine the native molecular weights of membrane proteins [18]. For example, Dickie and Weiner [19] were able to use sodium cholate to determine the minimal molecular weight of membrane-bound fumarate reductase of *E. coli* on a Sephacryl S-200 column. Since sodium deoxycholate is efficient in solubilizing the

mannitol permease from *E. coli* membranes [3], we employed chromatography on Sephacryl S-200 in the presence of this detergent to investigate subunit interactions in the nondenatured, active permease.

Extraction and chromatography of the permease in the presence of 0.25% deoxycholate resulted in two distinct peaks of activity corresponding to molecular weights of 130 (±10%) kDa and 65 (±5%) kDa. These correspond to the expected sizes of dimer and monomer respectively. However, incubation and elution of the mannitol permease in the presence of Pi favored the dimer while mannitol alone, and PEP-dependent phosphorylation of the mannitol permease, favored the monomer. These results are in complete agreement with those of Stephan and Jacobson [7] who had previously shown that under partially denaturing conditions, mannitol and phosphorylation of the protein decreased the amount of extractable dimer, whereas Pi stabilized the dimer.

From our results we can infer that the active permease must exist in at least two conformations, one which favors the monomer (or a less stable dimer) and one which favors the dimer (or a more stable dimer). The monomer is favored by PEP-dependent phosphorylation and/or mannitol binding, while the dimer is favored by conditions promoting phospho-exchange between mannitol and mannitol 1-phosphate (e.g., in the presence of Pi). These observations suggest (not necessarily exclusively) a kinetic model involving these two conformations of the permease shown in Figure 3. Not only does this model explain the effects of PEP-dependent phosphorylation or mannitol binding on subunit interactions (steps 1–4 in Fig. 3), but it also explains why transphosphorylation (reversal of steps 6, 5, and 3, followed by 3, 5, and 6 in the forward direction) is favored by stabilization of the dimer, since it is this form that is hypothesized to be required for phosphorylation of the enzyme by mannitol 1-phosphate. If this is true, then the dimer should either have a higher affinity for mannitol 1-phosphate or have a higher rate constant for phosphorylation of the protein by mannitol 1-phosphate (reversal of step 5, Fig. 3) than the monomer. However our kinetic results (Table II), show that Pi, which activates transphosphorylation and stabilizes the dimer, increases the apparent K_m for mannitol 1-phosphate in this reaction and that the activation effect is due rather to an increase in V_{max}. Thus, we conclude that the rate constant for phosphorylation of the permease by mannitol 1-phosphate is greater for the dimeric form than for the monomer, which would explain why the dimer is necessary for, or least more active in, the transphosphorylation reaction. The lack of a significant effect of Pi on the rate of the PEP-dependent

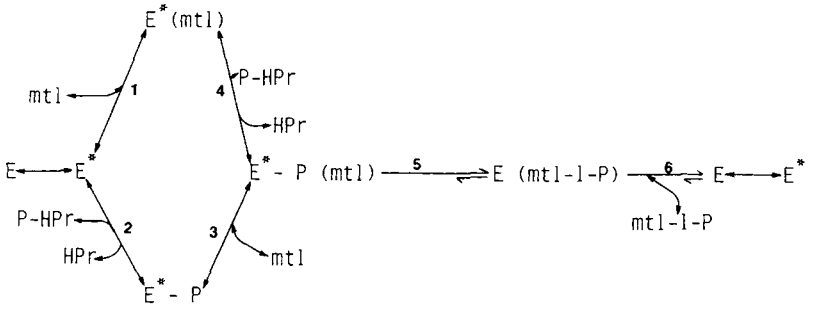

Fig. 3. A hypothetical kinetic model involving monomer and dimer conformations of the mannitol permease. See text for details. E = dimer; E* = monomer.

reaction can be explained either by the fact that the dimer appears to be stabilized by HPr, even in the absence of Pi [10], or that Pi stabilization of the dimer may not affect a rate limiting step in this reaction.

Very recently, Pas et al. [20] have shown that the mannitol permease is phosphorylated on two separate sites by phospho-HPr. Site I is the direct phospho-acceptor from phospho-HPr, while site II must be phosphorylated for phosphotransfer to occur between the permease and mannitol. Transfer of the phosphoryl group between site I and site II was inferred by these workers to be an intramolecular process. If this is true, then transphosphorylation should only require phosphorylation of site II by mannitol 1-phosphate, which we hypothesize to be favored by the dimer. Phosphorylation of site I by phospho-HPr, however, would not require the dimer and, in fact, appears to stabilize the monomer. It is during step 5 of Figure 3, then, in which the intramolecular transfer from site I to site II would occur. Since this step also involves a conformational change interconverting monomer and dimer, it is tempting to speculate that translocation of the hexitol across the membrane also occurs during step 5. Clearly, then, this step of the reaction sequence must actually consist of several substeps involving the different phosphorylated forms, and conformations, of the permease. The model presented in Figure 3 would also explain the apparent paradox that conditions favoring the PEP-dependent reaction (mannitol binding and PEP-dependent phosphorylation of the permease) favor the monomer, while the dimer has been inferred to be the minimum functional unit for the overall transfer of phosphate from PEP to mannitol catalyzed by the permease [9,10]. If the dimeric form is an obligatory intermediate in the overall reaction as shown in Figure 3, then this apparent paradox can be explained. It should also be pointed out, as we have done before [7], that the physical form of the mannitol permease in the membrane may always be a dimer, and that the monomer-dimer equilibrium observed in detergent solution may simply reflect the stability of this dimer under various conditions. In any case, however, these two (at least) conformational states of the protein appear to interconvert during one catalytic cycle of the permease as shown in Figure 3.

Further work will be necessary to establish the details of the model that we have presented. In particular, if the different phosphorylated forms of the mannitol permease can be separated for study, or separately identified in a mixture, then our hypothesis, that the phosphorylation of site I is differently affected by the conformational state of the protein than that of the site II, could be tested. Chemical modifications [20] and/or localized mutagenesis affecting only one of these sites will be useful for this purpose.

ACKNOWLEDGMENTS

We are grateful to Dr. R. Hausman for providing chromatographic equipment used during the course of this investigation. This research was supported by NIH grant GM 28226 from the National Institute of General Medical Sciences to G.R.J.

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