# Independent Folding of the Domains in the Hydrophilic Subunit IIAB$^{man}$ of the Mannose Transporter of *Escherichia coli*†

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ABSTRACT: The active form of the hydrophilic subunit (IIAB$^{man}$) of the mannose transporter of *Escherichia coli* is a homodimer of two 35-kDa subunits. Each subunit consists of two distinct domains, IIA and IIB, which can be separated by limited trypsin digestion. Separation of tryptic fragments yields monomers of IIB and dimers of IIA, which are active and stable. To test whether the domains fold as independent units, the effects of guanidine hydrochloride (GuHCl) and temperature on the structural stability of the intact IIAB$^{man}$ were compared with those of the isolated fragments. Equilibrium GuHCl-induced reversible unfolding, measured by circular dichroism and tryptophan fluorescence, showed a biphasic transition for intact IIAB$^{man}$ and monophasic transitions for each isolated fragment. The midpoint transitions of the isolated IIB and IIA fragments (at 1.0 and 2.3 M GuHCl) coincide with the first and second transitions of intact IIAB$^{man}$. Analytical ultracentrifugation studies suggested that dissociation precedes the unfolding of IIA. Thermal unfolding of IIAB$^{man}$, monitored by differential scanning calorimetry, showed two well-separated transitions near 52 and 95 °C which corresponded to the midpoint transitions of the isolated IIB and IIA fragments. The combined results demonstrate an independent stepwise unfolding of the domains in IIAB$^{man}$ as well as the absence of stabilizing interdomain interactions. The lack of interdomain interactions suggests an unrestricted domain motion. This may play an important role in the phosphoryl transfer reaction, which is catalyzed by the binding of IIAB$^{man}$ to a phosphoryl carrier protein HPr (via the IIA domain) and to the transmembrane subunits of the mannose transporter (via the IIB domain).

The mannose transporter of *Escherichia coli* is a protein complex consisting of a peripheral cytoplasmic subunit, IIAB$^{man}$,[1] and two membrane-spanning subunits, IIC$^{man}$ and IID$^{man}$. The three subunits together catalyze the transport and phosphorylation of mannose and related hexoses. Phosphorylated IIAB$^{man}$ is a catalytic intermediate in this process. The phosphoryl group is sequentially transferred from phosphoenolpyruvate to a sugar substrate via three cytoplasmic proteins of the bacterial phosphotransferase system (PTS): phosphoenolpyruvate → enzyme I → HPr → enzyme complex IIAB$^{man}$(IIC$^{man}$/IID$^{man}$) → mannose. Histidines are phosphorylated residues in all three proteins.

The subject of this study is the hydrophilic IIAB$^{man}$ subunit. It can be isolated as a complex with IIC$^{man}$ and IID$^{man}$ or as a soluble protein (Erni et al., 1987). The soluble form is a homodimer. Each monomer (35 kDa) consists of two structurally and functionally distinct domains, the N-terminal domain IIA (14 kDa) and the C-terminal domain IIB (20 kDa), which are linked by an Ala-Pro-rich flexible hinge. The role of IIAB$^{man}$ is to catalyze phosphoryl transfer from HPr to the sugar substrate. During this process both domains are transiently phosphorylated, IIA on His10 and IIB on His175. The IIA domain contains the sites for dimerization and interactions with HPr while the monomeric IIB domain binds to IIC$^{man}$/IID$^{man}$ (Erni et al., 1989). Limited trypsin digestion followed by column chromatography was used to produce the stable separated domains of the IIB monomers and IIA dimers (Erni et al., 1989). When mixed, they functionally complement each other.

The hydrophilic subunit IIAB$^{man}$ is involved in at least four types of protein–protein interactions. (i) With the IIC/IID complex via the IIB domain: the isolated IIB domain can bind to the membrane subunits IIC/IID and in this complex it catalyzes equilibrium phosphoryl exchange between Glc and Glc-6P (Erni et al., 1989; Q. Mao, unpublished results). (ii) With HPr through the IIA domain: the isolated IIA domain can be phosphorylated by HPr in the absence of the second domain (Erni et al., 1989). (iii) Interdomain interactions between IIA and IIB have not been well studied until now. Within IIAB$^{man}$ the IIB domain is phosphorylated by IIA. Although each isolated fragment can be phosphorylated in the absence of the second domain, though with lower efficiency (unpublished results), the presence of both IIA and IIB is required for the phosphorylation of the sugar substrate. (iv) Interdomain interaction between the IIA domains in the IIAB$^{man}$ homodimer. Isolated IIA is also a homodimer. Interactions (iii) and (iv) between IIA and IIB and between IIA itself are the subject of this study. If sufficiently strong interdomain interactions occur, they would be reflected in the degree of structural interdependence of the two domains. This

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[1] Abbreviations: GuHCl, guanidine hydrochloride; IIAB$^{man}$, hydrophilic subunit of the mannose transporter; IIA, amino-terminal domain of IIAB$^{man}$; IIB, carboxyl-terminal domain of IIAB$^{man}$; IIC$^{man}$ and IID$^{man}$, transmembrane subunits of the mannose transporter; PTS, phosphoenolpyruvate–sugar phosphotransferase system; HPr, histidine-containing phosphoryl carrier protein of the phosphotransferase system; CD, circular dichroism; DSC, differential scanning calorimetry; PGK, phosphoglycerate kinase.

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could be assessed by comparing the folding/unfolding transitions of integrated (within $IIAB^{man}$) and isolated IIA and IIB domains.

Since both domains of $IIAB^{man}$ can easily be isolated as stable functional fragments, $IIAB^{man}$ is an excellent candidate for testing the hypothesis of independent domain folding in multidomain proteins (Goldberg, 1969; Wetlaufer, 1973). Experimental observations of "single" as opposed to "multiple" unfolding transitions have been attributed to the specific interdomain interactions in multidomain single-chain proteins. A thermodynamic model was proposed for interpretation of differential scanning calorimetry data (Brandts et al., 1989), in which the contribution of interdomain interactions to the protein stability could be attributed to interaction free energy at the domain–domain interface. It is generally accepted that the cooperative unfolding of individual domains as a single unfolding unit and the absence of partially folded intermediates reflect strong interactions. Examples are heat denaturation of phosphoglycerate kinase (Griko et al., 1989; Freire et al., 1992) and arabinose binding protein (Fukada & Sturtevant, 1983), among others. Conversely, weak or absent interactions result in the independent unfolding of domains characterized by the presence of partially folded intermediates, in which some domains are still folded and the other already unfolded. Cold denaturation of phosphoglycerate kinase (Griko et al., 1989; Freire et al., 1992) and GuHCl and heat denaturation of γB-crystalline from calf eye lens (Rudolph et al., 1990; Mayr et al., 1994) illustrate such behavior. Thus, the extent to which domains interact determines the population of partially folded equilibrium intermediates (Freire et al., 1992; Freire & Murphy, 1991).

To assess the IIA–IIB and IIA–IIA interactions, the GuHCl- and temperature-induced conformational transitions of the separated and integrated IIA and IIB domains were compared. The degree of structural independence of the IIA and IIB domains in intact $IIAB^{man}$ is an indirect measure of the extent of interdomain interactions. The conformational transitions were monitored by circular dichroism, intrinsic fluorescence, analytical ultracentrifugation, and differential scanning calorimetry. Detailed analyses of the experimental results indicate that the interactions between the IIA and IIB domains are either absent or extremely weak and that dissociation of the IIA dimer precedes the unfolding.

## MATERIALS AND METHODS

*Protein Purification.* The wild-type form of $IIAB^{man}$ (encoded on plasmid pTSL2) was expressed in the *E. coli* strain WA2127ΔHIC as described elsewhere (Erni et al., 1987). $IIAB^{man}$ was purified from 6-L cell cultures according to the published procedure (Erni et al., 1987). The $NH_2$-terminal domain (IIA) and the COOH-terminal domain (IIB) were produced by limited tryptic digestion of $IIAB^{man}$ at a $IIAB^{man}$:trypsin molar ratio between 400:1 and 1000:1. The IIA and IIB fragments were separated by phosphocellulose chromatography and further purified by gel-filtration chromatography, as reported earlier (Erni et al., 1989). Protein concentrations of $IIAB^{man}$, IIA, and IIB were determined spectrophotometrically at 280 nm using extinction coefficients of 0.83, 1.42, and 0.49 $M^{-1}$ $cm^{-1}$, respectively.

*Reagents.* All experiments reported in this paper were carried out with protein samples extensively dialyzed at 4 °C against the standard buffer: 20 mM sodium phosphate, pH 7.4. High-purity guanidine hydrochloride was purchased from Sigma. All other chemicals were of the highest purity available.

*Unfolding–Refolding Studies.* Thermal denaturation was studied by DSC, and equilibrium GuHCl-induced unfolding was studied by fluorescence, far-ultraviolet CD, and analytical ultracentrifugation. To assure equilibrium conditions for each GuHCl concentration, protein–GuHCl mixtures were incubated for 2 h at 22 °C prior to the measurements. For refolding studies, protein samples were first fully denatured in 8 M GuHCl for 2 h at 22 °C and subsequently diluted with denaturing buffer to lower final concentrations of GuHCl (final protein concentrations were in the range 0.2–7.1 μM for fluorescence and 3–40 μM for CD and analytical ultracentrifugation measurements). Longer incubation times, up to 6 h, yielded the same results with both denaturation and renaturation experiments.

*Physical Measurements.* Fluorescence emission spectra (295-nm excitation) were recorded with an LS-5B Perkin-Elmer spectrofluorometer using a 5-mm path length cell. All spectra were corrected for solvent emission. The far-ultraviolet circular dichroism spectra were recorded on a Jasco spectropolarimeter (J500-A) using a 0.5-mm path length cell.

Scanning calorimetry were performed on a Microcal MC2-D instrument at a standard scan rate of 60 °C $h^{-1}$ with buffer in the reference cell. Protein samples, diluted with standard buffer to 1.5–3.5 mg $mL^{-1}$, were scanned in the range from 20 to 110 °C and rescanned to check reversibility. A buffer baseline correction and concentration normalization was applied and the data were analyzed with the standard software package for protein unfolding (Microcal ORIGIN), yielding the transition midpoint temperature ($T_m$), the calorimetric enthalpy ($\Delta H_{cal}$), and the van't Hoff enthalpy ($\Delta H_{VH}$).

Centrifugation measurements were performed in the Beckman (Model XLA) analytical ultracentrifuge. Sedimentation velocity runs of the native proteins were carried out at 52 000 rpm at 20 °C. The apparent molecular masses of homodimeric proteins (70 and 28 kDa) were determined by the method of sedimentation equilibrium in the presence and absence of GuHCl with the aim to define the range of GuHCl concentration where the dissociation of dimers occurs. The 12-mm Epon-filled charcoal double-sector cells were used. Two sectors were filled with 0.07 mL of protein solutions and solvent, respectively, and FD-43 bottom fluid. In the range between 0 and 6 M GuHCl the rotor speed was varied between 24 000 and 36 000 rpm for the 28-kDa protein and between 13 000 and 22 000 rpm for the 70-kDa protein. Under these conditions, which approach those of the short column described by Yphantis (1960), it was possible to fit the experimental data with a single exponential function along the entire range of GuHCl concentration studied. The absorption data were analyzed as a function of the square radii (ln $A$ against $r^2$ plots) and the apparent molecular masses were evaluated with a computer program similar to that of Chernyak (1982) using a floating baseline algorithm. A partial specific volume of 0.73 $cm^3/g$ was assumed in all calculations. The densities of GuHCl buffers were taken from Kawahara and Tanford (1966).

*Activity Assay.* Sugar phosphorylation of $IIAB^{man}$ and of equimolar mixtures of tryptic fragments were assayed by the ion-exchange method of Kundig and Roseman (1971) as specified by Stolz et al. (1993).

*Analysis of GuHCl-Induced Unfolding Curves.* The fraction of unfolded protein ($f_u$) was calculated from the experimental transition curves by using the equation $f_u = (y_f - y_{obs})/(y_f - y_u)$, where $y_{obs}$ is the observed variable parameter (e.g., circular dichroism, fluorescence emission) and $y_f$ and $y_u$ are values characteristic of the folded and unfolded confor-

mations. For the calculation of $f_u$ values in the transition region, $y_f$ and $y_u$ were extrapolated from the linear portions of the pre- and posttransitional regions of the unfolding curve into the transition region.

Thermodynamic parameters characteristic of the unfolding of the separated IIB domain were estimated from the $f_u$ dependence on GuHCl concentration. The two-state model

$$M_f \overset{K_u}{\rightleftharpoons} M_u$$

was assumed for the unfolding reaction, where $M_f$ and $M_u$ stand for folded and unfolded monomer. The equilibrium constant of unfolding ($K_u$) can be expressed in terms of the fraction of unfolded protein $f_u$:

$$K_u = \frac{[M_u]}{[M_f]} = \frac{f_u}{1 - f_u} \quad (1)$$

For the two-state unfolding model, the free energy of unfolding ($\Delta G = -RT \ln K_u$) was shown to be a linear function with respect to denaturant concentration (Pace, 1986):

$$\Delta G = \Delta G(H_2O) - m[\text{GuHCl}] = -RT \ln K_u \quad (2)$$

where $\Delta G(H_2O)$ is the free energy in the absence of GuHCl, $m$ is the fitting parameter that reflects the steepness of the unfolding transition, [GuHCl] is the concentration of GuHCl, and $R$ and $T$ are the gas constant and the absolute temperature, respectively. The values of $\Delta G(H_2O)$ and $m$ can be obtained directly from the unfolding curve ($f_u$ vs GuHCl) by fitting the experimental data for IIB using a nonlinear least-squares program to the rearranged eq 1

$$f_u = \frac{K_u}{K_u + 1} \quad (3)$$

where $K_u$ is a function of $\Delta G(H_2O)$, $m$, and [GuHCl] (see eq 2).

## RESULTS

*Phosphotransferase Activity.* An equimolar mixture of isolated IIA and IIB exhibits 15% sugar phosphorylation activity relative to that of the native IIAB$^{man}$. Activity could not be used as a means to monitor denaturation by GuHCl due to the need for the presence of enzyme I, HPr, and IIC$^{man}$/IID$^{man}$ in the assay. Any of these additional PTS proteins may become denatured in the presence of GuHCl. However, 80–90% activity of IIAB$^{man}$ and of the IIA and IIB domains could be recovered after removal of 8 M GuHCl by dialysis against standard buffer at 4 °C for 36 h, confirming the overall reversibility shown by circular dichroism and tryptophan fluorescence.

*GuHCl-Induced Transitions Monitored by Spectroscopic Methods.* The equilibrium unfolding of IIAB$^{man}$ and of the isolated tryptic fragments IIA and IIB were studied at neutral pH by far-UV CD and tryptophan fluorescence, which reflect changes in the secondary and local tertiary structure that occur during the transition. There are four tryptophan residues per IIAB$^{man}$ monomer, three in the IIA domain (W12, W33, and W69) and one in the IIB domain (W182). The emission spectra of native IIAB$^{man}$ and the IIA and IIB domains (excitation at 295 nm) have emission maxima centered at 343, 347, and 333 nm, respectively. Thus, the tryptophan residues can be used as reporters of GuHCl-induced local conformational changes occurring in each domain and in the intact IIAB$^{man}$.

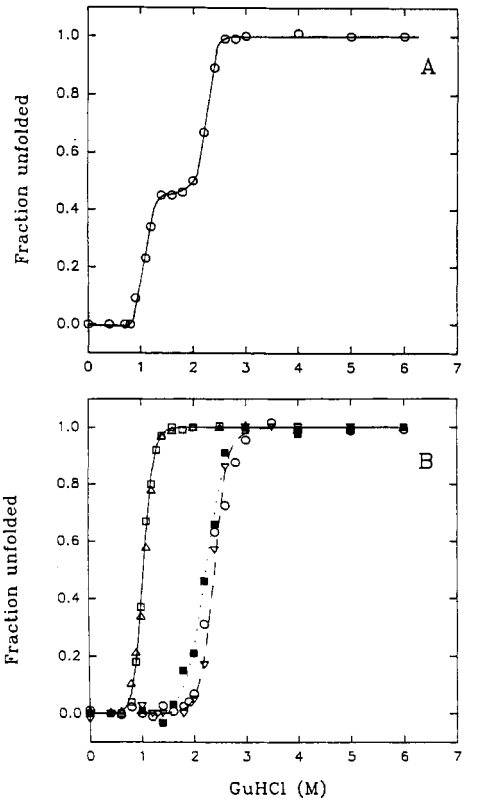


FIGURE 1: GuHCl-induced unfolding of intact IIAB$^{man}$ (A) and of the isolated tryptic fragments (B) in 20 mM sodium phosphate, pH 7.4, at 22 °C. Normalized data are reported as the fraction of the unfolded protein. All data were normalized to the difference between the linear changes observed above and below the transition region. (A) Circular dichroism at 222 nm of IIAB$^{man}$ (2.9 μM) (○). (B) Circular dichroism at 222 nm of IIB (5 μM) (□) and IIA (7.1 μM) (▽); fluorescence emission at 333 nm of IIB (2 μM) (△) and fluorescence maximum shift of IIA (7.1 μM) (○); apparent molecular weight of IIA (7.1 μM) (■). The solid line shown for IIB was computed from eq 3; the dashed and dotted lines shown for IIA are the smoothed curves drawn through the data points.

Figures 1A and 2A show a biphasic unfolding process of intact IIAB$^{man}$ measured by CD at 222 nm and fluorescence emission at 333 nm, respectively. Two sharp, well-separated transitions near 1.0 and 2.3 M GuHCl were shown by both methods. This suggests the presence of at least one intermediate state in the unfolding process. The intermediate state appeared to be stable at about 1.5 M GuHCl and possessed about 50% of the native CD amplitude and 75% of the fluorescence emission. Beyond 3 M GuHCl, IIAB$^{man}$ is extensively randomized as shown by the drastic reduction of the secondary structure (8% of the CD signal of the native protein) in the far-UV CD spectrum (result not shown). If the fluorescence intensity of IIAB$^{man}$ was monitored at 344 nm, near the emission maximum characteristic of the IIA fragment (347 nm), only a single transition near 2.3 M GuHCl was observed (result not shown) which corresponded to that of the isolated IIA fragment (see below). The absence of the first transition at 1 M GuHCl is due to the negligible contribution of the buried W182 ($\lambda_{max}$ = 333 nm) to the overall fluorescence at 344 nm. The unfolding of IIAB$^{man}$ was found to be fully reversible by both methods.

The unfolding transition of the isolated IIB fragment, monitored by CD at 222 nm and fluorescence emission at 333 nm, is monophasic (Figure 1B) with midpoints of transitions identical to those observed with the integrated domain (Figures 1A). The fluorescence red shift from 333 to 353 nm indicated exposure of W182 to solvent upon denaturation (results not

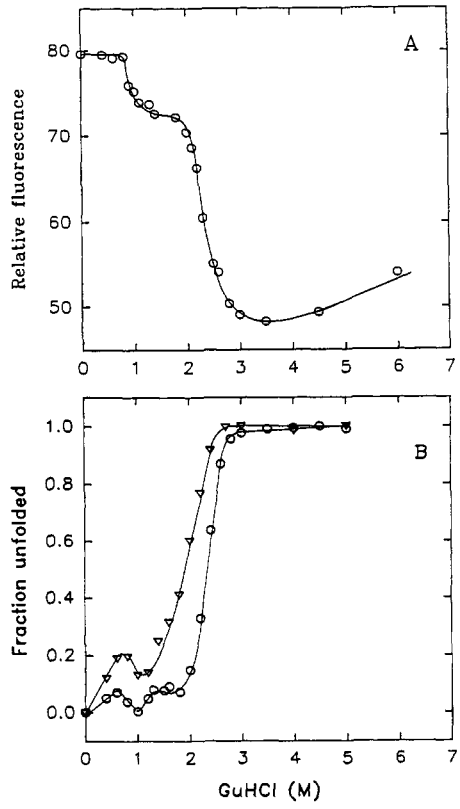


FIGURE 2: Effect of GuHCl on the fluorescence emission of IIAB$^{man}$ and IIA. Excitation was at 295 nm. Experiments were carried out in 20 mM sodium phosphate, pH 7.4, at 22 °C. (A) Relative fluorescence of intact IIAB$^{man}$ (0.7 $\mu$M) at 333 nm. (B) Relative fluorescence of IIA at 347 nm expressed as the fraction of unfolded protein. The data were normalized to the difference between 0 and 5 M GuHCl. Symbols (○) and (▽) in (B) represent protein concentration of 2.5 and 0.25 $\mu$M, respectively.

shown). The normalized CD and fluorescence signals shown in Figure 1B follow identical reversible transitions, between 0.8 and 1.5 M GuHCl, with midpoints near 1.0 M GuHCl. This suggests that the unfolding of the IIB domain is a cooperative transition which proceeds from folded ($M_f$) to unfolded ($M_u$) monomer via a two-state mechanism:

$$M_f \overset{K_u}{\rightleftharpoons} M_u$$

Fitting of experimental data to this model showed a good agreement between the observed and predicted transition curves, supporting the choice of a two-state model (Figure 1B). The free energy $\Delta G(H_2O)$, describing the conformational stability of the protein at 25 °C in the absence of denaturant, was calculated to be 6.5 kcal mol$^{-1}$. The same values of $\Delta G(H_2O)$ were obtained from a linear extrapolation of $\Delta G$ versus [GuHCl] (see eq 2) according to Pace (1986).

The GuHCl-induced unfolding of the isolated IIA domain, followed by CD (222 nm) and by the shift of the fluorescence emission maximum (from 347 to 353 nm), is fully reversible. The normalized signals from both methods exhibit similar sigmoidal transitions with midpoints near 2.3 M GuHCl, indicating cooperative unfolding of the IIA domain (Figure 1B). These transitions are not coincident with that obtained by molecular weight measurements in the analytical ultracentrifugation (see below).

The unfolding transition of IIA recorded by the fluorescence intensity (at 347 nm) shows nonlinear changes in the pretransitional region (Figure 2B) which are absent in the sigmoidal transition shown by circular dichroism and fluorescence maximum shift (Figure 1B). This nonlinearity may be, at least in part, explained by the heterogeneity of the tryptophan fluorescence, which was demonstrated in independent studies of Trp mutants (Z. Markovič-Housley, manuscript in preparation). It was shown that in the native enzyme W12 is exposed to solvent ($\lambda_{em}^{max}$ = 347 nm) while W33 and W69 are partially buried ($\lambda_{em}^{max}$ = 341 nm). The fluorescence of W69 is almost completely quenched, both in IIAB$^{man}$ and in the isolated IIA domain. The unfolding transitions recorded with tryptophan mutants lacking Trp 69 (W69F and W12,69F) do not show the small fluorescence increase near 1.0 M GuHCl of the wild type. This allows us to assign the small intensity increase near 1.0 M GuHCl to a gradual unquenching of Trp 69 rather than to more complex molecular rearrangements.

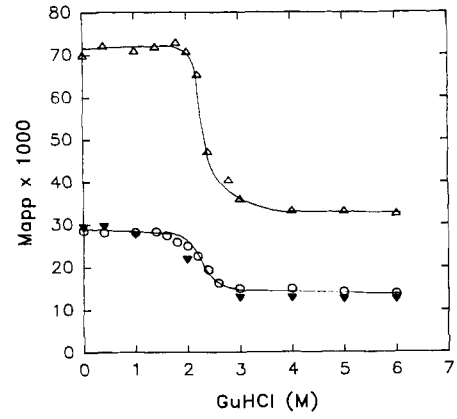


FIGURE 3: Effect of GuHCl on the apparent molecular weight ($M^{app}$) of dimeric IIAB$^{man}$ (△) and isolated IIA (○) in 20 mM sodium phosphate, pH 7.4, determined by analytical ultracentrifugation at 22 °C. Protein concentration was 7.1 $\mu$M. ▼ represents the values of $M^{app}$ corrected for a possible dependence of $\bar{v}_2$ on the concentration of GuHCl (for details see Results).

*Studies of GuHCl-Induced Dissociation of Dimeric Proteins.* The results obtained with CD and fluorescence spectroscopy do not offer any information as to the importance of the monomer interaction in the IIAB$^{man}$ and IIA dimers. Since complete unfolding of noncovalent dimers must involve dissociation into monomers, the question is whether subunit dissociation occurs prior to or concomitant with the GuHCl-induced unfolding of the IIA domain. To answer the above question, two kinds of experiments were performed: analytical ultracentrifugation was used to measure the apparent molecular mass as a function of GuHCl, and different techniques were applied to study the effect of protein concentration on the unfolding/dissociation transitions.

*Analytical Ultracentrifugation.* IIAB$^{man}$ and IIA were analyzed by analytical ultracentrifugation as a function of GuHCl concentration. Sedimentation velocity runs indicated homogeneous protein solutions for IIAB$^{man}$ and IIA characterized by sedimentation coefficients of 3.7 and 2.6 S, respectively, at 0 M GuHCl. Molecular weight measurements by sedimentation equilibrium ($M^{app}$) showed that IIAB$^{man}$ and IIA are dimers of 70 and 28 kDa at all GuHCl concentrations below 1.8 M (Figure 3). With both proteins, further increases in GuHCl concentration up to 3 M resulted in a decrease of the apparent molecular weight, due to an increase in the fraction of the monomeric species in the transition region. Above 3.0 M GuHCl only the monomers of IIAB$^{man}$ (35 kDa) and IIA (14 kDa) were observed. The midpoint of the dimer to monomer transition is between 2.2 and 2.3 M GuHCl for both IIAB$^{man}$ and the isolated IIA domain.

In the calculation of the apparent molecular weights the assumption that the partial specific volume, $\bar{v}_2$, is constant is not in agreement with the theory of the thermodynamics of multicomponent solutions as developed by Casassa and Eisenberg (Casassa & Eisenberg, 1961, 1964; Eisenberg, 1981). Experimental data on denaturation of various proteins by GuHCl indicate that the partial specific volume in 5–6 M GuHCl is lower by about 0–0.03 cm$^3$/g than in the native state [see review by Durchschlag (1986)]. Few works have reported on changes for the whole range between 0 and 6 M GuHCl (Durchschlag & Jaenicke, 1982; Reisler & Eisenberg, 1969; Reisler et al., 1977). Although the extent of changes in the partial specific volume may vary from protein to protein, there is a general agreement that $\bar{v}_2$ decreases with an increase in GuHCl concentration. In order to obtain an approximate estimate of the changes in $M^{app}$ which would be caused by a GuHCl-induced decrease of $\bar{v}_2$, we have assumed that $\bar{v}_2$ of IIA varies with the concentration of GuHCl in a manner similar to that reported for bovine serum albumin (Durchschlag & Jaenicke, 1982). The corrected values of $M^{app}$, shown in Figure 3, indicate that the overall trend is a shift of the transition curve to a lower GuHCl, and so the noncoincidence of the dissociation and unfolding transitions is more accentuated. Although the above estimates support the observation that dissociation precedes unfolding, the exact extent of "nonsimultaneity" of the unfolding and dissociation curves is difficult to determine precisely in the absence of an experimental value of $\bar{v}_2$ as a function of GuHCl concentration.

Comparison of the normalized $M^{app}$ transition of IIA with those monitored by CD and fluorescence emission (Figure 1B) indicated that at a concentration of 7.1 $\mu$M the dissociation of the IIA dimers closely precedes the unfolding, which suggests the presence of the intermediate(s) of dissociation and unfolding. If both folded monomers ($M_f$) and dimers ($D_f$) are significantly populated in the transition region, the simplest mechanism which may describe the overall unfolding process is the three-state model $D_f \rightleftharpoons 2M_f \rightleftharpoons 2M_u$. Since the dissociation and unfolding are bi- and unimolecular processes, respectively, only the former reaction is expected to be dependent on protein concentration. If the dissociation and unfolding are well-separated processes, then the variation of protein concentration should affect the transition curve only at the concentrations of denaturant which are responsible for dimer dissociation. To test this possibility, the CD, fluorescence, and $M^{app}$ transitions were studied at various protein concentrations.

*Effect of Protein Concentration on the Unfolding/Dissociation Transitions.* The unfolding transitions of IIA shown in Figure 1B were not affected by an increase of protein concentration above 3 $\mu$M (up to 40 $\mu$M for CD and $M^{app}$ and up to 7.1 $\mu$M for fluorescence measurements). Unfortunately, below 3 $\mu$M only the fluorescence emission could be measured since the CD and $M^{app}$ signals became unreliably low. The decrease of the protein concentration from 2.5 to 0.25 $\mu$M affected the fluorescence intensity transition along the entire range of denaturant concentration (Figure 2B). The overall transition occurred in three phases: (1) a decrease of fluorescence intensity between 0 and 0.6 M GuHCl, which is more pronounced at a protein concentration of 0.25 $\mu$M than at 2.5 $\mu$M; (2) a small increase of fluorescence emission between 1.0 and 1.2 M GuHCl; and (3) a major decrease of fluorescence from 1.6–1.8 M GuHCl to the plateau region above 3 M GuHCl. To simplify further discussion we shall ignore the increase in fluorescence between 1.0 and 1.2 M GuHCl (second transition), which was shown to be due to the unquenching of Trp 69 (see above), and assume that the overall unfolding consists of two transitions (first and third) separated by a plateau. Thus, the major changes produced by a 10-fold dilution of protein concentration are a pronounced decrease of fluorescence intensity between 0 and 0.6 M GuHCl, a shift of the midpoint of the major transition from 2.3 to 2.0 M GuHCl, and a decreased intensity within the plateau region. Unfortunately, the information on the quaternary and secondary structures cannot be obtained at a protein concentration of 0.25 $\mu$M because the measured signals becomes insignificant with respect to the background noise. This limits a detailed characterization of the intermediate(s) species. Nevertheless, certain deductions can be made and these are discussed below.

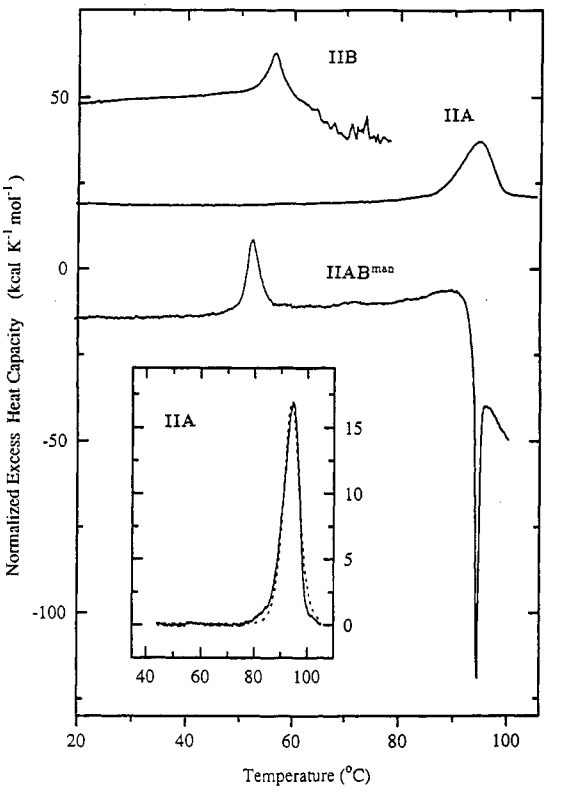


FIGURE 4: Temperature dependence of the molar heat capacity of intact IIAB$^{man}$ (1.7 mg mL$^{-1}$) and its isolated fragments IIB (1.7 mg mL$^{-1}$) and IIA (2.7 mg$^{-1}$ mL). Proteins were dissolved in 20 mM sodium phosphate, pH 7.4. Heating rate was 60 °C h$^{-1}$. The inset shows the deconvolution of the heat capacity function of the IIA fragment. (—) Experimental values; (- - -) calculated values assuming a single cooperative transition.

*Temperature-Induced Unfolding Monitored by Differential Scanning Calorimetry.* The excess heat capacity profiles ($C_p$) as a function of temperature are shown for IIAB$^{man}$ and its tryptic fragments, IIB and IIA, in Figure 4. Thermal denaturation transitions of the isolated IIA and IIB fragments are characterized by single heat absorption peaks with midpoint temperatures near $T_m$ = 94 and 56 °C, respectively. The uncommonly sharp transition of IIB, indicating highly cooperative unfolding, is followed by noisy, exothermic baseline artifacts which are caused by protein precipitation after unfolding. While IIB denatures irreversibly, the unfolding of the IIA fragment was found to be reversible as shown by the reappearance of the heat absorption peak (about 70%) upon reheating of the cooled solution. For the intact IIAB$^{man}$, two well-separated transitions were observed. The midpoint of the first transition at 52 °C is similar to that obtained with the isolated IIB fragment. Unfortunately, once this domain

is unfolded, the protein starts to aggregate and the second transition at about 95 °C results in massive exothermic precipitation. Nevertheless, this transition is convincing and reproducible and occurs at the same temperature as that of the isolated IIA fragment. The finding that the transition temperature of the isolated IIB fragment is slightly higher (56 °C) compared with that observed in intact IIAB$^{man}$ (52 °C) may be due to IIB precipitation, or it may be indicative of negative cooperativity between the N- and C-terminal parts of dimeric IIAB$^{man}$. Similar behavior was observed with λ Cro repressor (Griko et al., 1992) and with enzyme I of *Salmonella typhimurium* (LiCalsi et al., 1991).

The thermodynamic information could only be obtained for the reversible transition of the IIA domain. The calorimetric enthalpy, $\Delta H_{cal}$, was determined from the area of the heat absorption peak, while the van't Hoff enthalpy, $\Delta H_{VH}$, was estimated from the sharpness of the heat absorption peak by assuming that the peak represents a two-state process. The thermal denaturation of IIA is characterized by values of $\Delta H_{cal}$ = 135 kcal mol$^{-1}$ and $\Delta H_{VH}$ = 136 kcal mol$^{-1}$. The excellent agreement of these two values indicates that the N-terminal domain of IIAB$^{man}$ unfolds in a two-state process. For IIB and IIAB$^{man}$ a more rigorous treatment of the irreversible denaturation with different protein concentrations and scanning rates is needed for accurate thermodynamic characterization of the unfolding process (Freire et al., 1990).

## DISCUSSION

Three questions have been addressed in these studies in an effort to understand what governs the folding of the two-domain homodimeric protein IIAB$^{man}$: (1) Do individual domains fold independently and reversibly and what is the extent of interdomain interactions? (2) What are the stabilities of each domain? (3) What role do the intersubunit interactions play in the stabilization of the final folded structure?

In answer to the first question, our studies provide direct evidence of independent folding of IIA and IIB domains in homodimeric IIAB$^{man}$. This conclusion was reached by comparing the unfolding/folding transitions of native IIAB$^{man}$ with those of its separated tryptic fragments. GuHCl-induced equilibrium unfolding, monitored by CD, fluorescence emission, and analytical ultracentrifugation, was fully reversible for intact IIAB$^{man}$ and isolated tryptic fragments, whereas the heat denaturation, monitored by DSC, was reversible only for the IIA fragment. Both the GuHCl- and temperature-induced folding studies demonstrated that the midpoints of the monophasic transitions of the separated IIB (1.0 M GuHCl; 56 °C) and IIA (2.3 M GuHCl; 95 °C) fragments coincide with the first and second transition in the biphasic denaturation profile of intact IIAB$^{man}$. These findings allow us to conclude that (i) isolated IIA and IIB are capable of folding as separate entities to achieve the conformation and stability as present in the intact IIAB$^{man}$; (ii) there are no detectable interdomain interactions which contribute to domain stability; and (iii) the biphasic transition of intact IIAB$^{man}$ reflects a stepwise unfolding of two cooperative folding units; that is, the first transition represents the unfolding of the C-terminal IIB monomers and the second represents the unfolding of the N-terminal IIA dimer. The simplest mechanism that could account for the unfolding reaction of IIAB$^{man}$ is N (native dimers) ⇌ I (dimeric intermediate) ⇌ U (unfolded monomers). The only significantly populated intermediate is partially folded dimer, consisting of the unfolded IIB domain and the folded IIA domain. The second unfolding transition from I to U is coupled with dimer dissociation, as shown by analytical ultracentrifugation.

The biphasic transition of IIAB$^{man}$ and the presence of partially folded intermediate is in agreement with weak or absent interdomain interactions. Many two-domain proteins show similar behavior, indicating that the stabilization of domains is mainly due to intra- rather than interdomain interactions [see review by Jaenicke (1987)]. The $\beta_2$ subunit of tryptophan synthase is another two-domain homodimeric protein that was also shown to exhibit stepwise independent unfolding of domains, consistent with weak or nonexistent interdomain interactions (Zetina & Goldberg, 1980). However, the absence of stabilizing interdomain interactions in the $\beta_2$ subunit was shown to be caused by a denaturant-induced uncoupling of the domains which occurs at low concentration of GuHCl (0.4 M), where the nicked $\beta_2$ dimer first dissociates into the folded domains and only then do the domains start to unfold at higher GuHCl concentrations. In IIAB$^{man}$ the lack of interdomain interactions seems to be an inherent property of the native structure rather then an artifact of a denaturing agent since the independent unfolding of domains was also demonstrated with temperature-induced denaturation in the absence of GuHCl.

Concerning the second question, considerably higher stability of IIA, as compared to IIB, is shown by the midpoints of the unfolding transitions induced by GuHCl (2.3 vs 1.0 M) and temperature (95 vs 56 °C). This is consistent with a higher proteolytic stability of IIA relative to IIB and an overall more compact structure of IIA (Erni et al., 1989). The difference in stability between IIA and IIB may lie in their secondary structure (50% versus 25% α-helical content). Likewise, the stability of IIA may be enhanced by intersubunit interactions, as observed for many oligomeric proteins (Jaenicke, 1987). This possibility is discussed below.

In many oligomeric proteins, unfolding and dissociation run in parallel and so prevent separate study of these two processes (Jaenicke, 1991). The extent of coupling between the dissociation and unfolding depends on the protein itself as well as on the experimental conditions. For dimeric proteins there are two extreme cases: (1) Both processes occur simultaneously in a concerted two-state reaction ($D_f \rightleftharpoons 2M_u$) from folded dimer ($D_f$) to unfolded monomer ($M_u$), such that no intermediates are sufficiently populated in the transition region. For example, the mutual stabilization of two unstable monomers in the dimeric DNA-binding protein λ Cro causes the simultaneous dissociation and unfolding of this protein (Pakula & Sauer, 1989). (2) If the processes of dissociation and unfolding are uncoupled, the intermediates of dissociation and unfolding exist in a defined concentration range of the dissociating agent. The simplest model which may describe the overall unfolding process is a sequential bi-unimolecular reaction of the type $D_f \rightleftharpoons 2M_f \rightleftharpoons 2M_u$. The dimeric aspartate aminotransferase from *E. coli* is an example where the dissociation of dimers (0.5 M GuHCl) precedes the unfolding of monomers (1.7 M GuHCl) (Herold & Kirschner, 1990).

The results reported here show that the reversible unfolding of IIA does not follow a simple two-state reaction. Although the transitions measured by circular dichroism and tryptophan fluorescence are well superimposable, they do not coincide with the transition observed with analytical ultracentifugation ($M^{app}$), which suggests the presence of equilibrium intermediate(s). Further support comes from the dependence of the fluorescence transition on protein concentration (Figure 2B). It was observed that the 10-fold dilution of protein concentration affected the fluorescence transition at the GuHCl concentrations within and below a major transition, resulting in a pronounced multiphasic transition profile. This supports

the conclusion that certain intermediate(s) must be present and thus the purely bimolecular mechanism of the type $D_f \rightleftharpoons 2M_u$ is excluded. On the other hand, the shift of the midpoint transition from 2.3 to 2.0 M GuHCl clearly rules out a purely unimolecular unfolding mechanism from folded to unfolded monomers ($M_f \rightleftharpoons M_u$) and further suggests that the IIA dimers must be significantly populated in the transition zone. Although a detailed analysis of the unfolding mechanism is not possible by equilibrium methods only, the simplest explanation which is consistent with these experimental observations is the following. At sufficiently high concentrations of IIA the equilibrium of dissociation is shifted toward the dimer so that the major components present in the transition region are the folded dimers and unfolded monomers. Under these conditions the overall unfolding process approaches a bimolecular two-state behavior ($D_f \rightleftharpoons 2M_u$) which is concentration dependent. This explains the observed shift of the midpoint transition from 2.3 to 2.0 M upon 10-fold dilution of protein concentration. In terms of the energetics of unfolding it is conceivable that the higher stability observed at the higher IIA concentration (2.5 $\mu$M) may be attributed to the interaction free energy at the dimer interface in addition to the free energy of monomer unfolding. As the protein concentration decreases, the equilibrium of dissociation is increasingly shifted toward the monomers. Consequently, both the folded dimers and monomers are present in the solution and the overall unfolding process may be described by a three-state model ($D_f \rightleftharpoons I \rightleftharpoons M_u$), consistent with the multiphase behavior observed at low concentrations. It would be interesting to know whether the transition observed between 0 and 0.6 M GuHCl reflects the dissociation of dimers, analogous to that reported for the $\beta_2$ subunit of tryptophan synthase (Zetina & Goldberg, 1980). Unfortunately, the lack of information about the quaternary and secondary structure at a protein concentration of 0.25 $\mu$M prevented a more detailed characterization of the intermediate(s).

The biological necessity for the dimeric structure of IIA and IIAB$^{man}$ is not evident. No dimers were observed with functionally homologous domains of other PTS proteins (Lolkema et al., 1993). Recently, complementation studies with various mutants showed that monomer exchange occurs between the dimers of various inactive mutants and the wild type, indicating that at the low protein concentrations used (80 nM) dimer dissociation must occur. Furthermore, the observation that the phosphoryl transfer in the IIAB$^{man}$ dimer occurs mainly between domains on the same subunit suggests that dimers are not very important for phosphoryl transfer (Stolz et al., 1993). It is conceivable that the joint subunits, which probably exist at high protein concentrations *in vivo*, provide more efficient phosphate transfer and possibly the binding site for phospho carrier protein HPr.

With regard to the biological role of the hinge in IIAB$^{man}$, we have at the beginning of this study anticipated the possibility that the hinge may mediate stabilizing interdomain interactions analogously to those reported for phosphoglycerate kinase (PGK) (Banks et al., 1979). In PGK, selected residues in the $\alpha$-helical hinge are directly involved in stabilizing interdomain interactions (Banks et al., 1979), which are responsible for a two-state cooperative unfolding of domains (Calderon et al., 1985; Griko et al., 1989). The biphasic unfolding of IIAB$^{man}$ and the demonstration that the stabilities of integrated and isolated IIA and IIB domains are the same clearly indicate that the hinge in IIAB$^{man}$ does not contribute to domain stabilization. Similar behavior was observed with $\gamma$B-crystalline, which is a single-chain protein consisting of two homologous domains joined by a flexible linker. Weak interdomain interactions in $\gamma$B-crystalline, seemingly independent of the hinge, are reflected in the biphasic transition profile and partially folded intermediate (Mayr et al., 1994).

An insight into the possible function of the hinge may be gained by examining the biological role of IIAB$^{man}$. During phosphate transfer the hydrophilic IIAB$^{man}$ acts as a part of the multienzyme complex in which IIA is bound to phospho carrier protein HPr and IIB to the membrane-bound subunits IICD$^{man}$. The phosphoryl group released by HPr is accepted by His10 in the IIA domain and subsequently transferred to His 175 in the IIB domain and hence to the sugar. Thus, the two histidine residues are probably in close proximity when the phosphoryl group is catalytically transferred. We have demonstrated that there are no detectable interdomain interactions under nonphosphorylating conditions *in vitro* which could bring the two domains together. Assuming that this observation applies also under phosphorylating conditions *in vivo* and *in vitro*, some conformational change is necessary to bring the two domains close to each other. A "hinge-bending" conformational change reported for phosphoglycerate kinase (Banks et al., 1979) and local "stiffening" of the hinge as reported for Ala-Pro-rich linker polypeptides (Radford et al., 1989) are two examples of hinge motions. Binding of IIAB$^{man}$ to the membrane complex IIC$^{man}$/IID$^{man}$ and to HPr inside the multienzyme complex could conceivably trigger the hinge motion *in vivo*. The observation that an equimolar mixture of the separated IIA and IIB fragments exhibits only 15% of the activity characteristic of native IIAB$^{man}$ suggests that the hinge is important for sugar phosphorylation. However, it is not clear whether the role of the hinge is to provide high local concentrations of domains required for activity or to perform specific conformational change. In either case, it seems that the function of the 20-residue-long flexible interdomain linker is to allow the IIA and IIB domains to move and interact with other PTS proteins as well as to adopt an orientation and proximity which facilitate catalytic transfer of phosphoryl groups. We thus conclude that independence of domain movement (IIA and IIB) must be an important factor for the biological function of IIAB$^{man}$. This independence is manifested in nonexistent interdomain interactions and in the conformational flexibility of the interdomain linker.

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