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Denaturation of a Membrane Transport Protein by Urea: The Erythrocyte Anion Exchanger

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Summary. Chloride equilibrium exchange was measured in the presence of intracellular and extracellular urea, several different alkylureas and thiourea. Urea half-inhibited Cl exchange at about 2.5 M, but the other, less polar analogs had significantly higher potencies; e.g., butylurea half-inhibited at about 60 mM. Onset and reversal of inhibition occurred within less than 2 sec. The inhibition exhibited no obvious sigmoidal dependence on urea concentration, and at low concentrations dimethylurea was a noncompetitive inhibitor of Cl exchange. However, at higher concentrations the Dixon plots were curved upward and a Hill analysis of the dimethylurea data yielded a Hill coefficient of at least 1.5. When present on only one side of the membrane, the slowly penetrating thiourea inhibited Cl exchange with a higher potency from the outside of the cell. Cl/Br exchange was inhibited less under conditions of self-inhibition of anion exchange than in the absence of self-inhibition. These data indicate that the ureas inactivate the anion transporter by a reversible denaturation process, and that the function of the anion transport mechanism may be more sensitive to small perturbations of protein structure than are spectroscopically derived structural parameters.

Key Words band 3 protein · anion exchange · denaturation · urea · self-inhibition

Introduction

The anion transport protein of the erythrocyte is the major transmembrane protein of the red cell membrane. Due to its abundance and the easy availability of erythrocytes it has become a model for transport as well as protein chemical studies (*see* Passow, 1986). The sequence of band 3 has recently been deduced from the messenger-RNA of murine erythrocytes (Kopito & Lodish, 1985). It possesses two parts of different properties: the major segment (55 kilodalton) contains alternating hydrophilic-hydrophobic sequences typical for a membrane-spanning protein, and the smaller segment (41 kD) is very polar and is probably dissolved in the cytoplasm. The anion transport kinetics have been examined in a large number of studies of monovalent and divalent anion transport (for reviews *see* Knauf, 1979; Passow, 1986). The exchange transport kinetics are those of a single-site, alternating-access carrier mechanism that mediates very high rates of monovalent anion exchange but much slower rates of net transport. Net transport, on the other hand, appears not to obey carrier kinetics but can, to a large degree, be described by a mechanism that resembles that of ionic movement through a very narrow channel-like structure (Fröhlich, 1984).

Most of the kinetic studies on anion transport have been performed with unperturbed band 3 in its native environment, that is either in intact cells or in resealed ghosts. It is also possible now to extract the protein from the membrane into detergent systems and to reconstitute it into artificial lipid vesicles, without a major loss of function and with apparently similar kinetic characteristics (Scheuring et al., 1986). While the protein retains its mainly dimeric state as well as certain inhibitor binding characteristics in mild nonionic detergents (Werner & Reithmeier, 1985; Scheuring et al., 1986), strong detergents such as SDS[1] and other denaturants such as urea or guanidine disrupt its structure and abolish its binding capacity for the stilbene derivative DBDS (Boodhoo & Reithmeier, 1984). Surprisingly, when the denaturant is removed, the protein is able to recover not only from exposure to urea or guanidine but also from mild SDS treatment both of which had dissociated the dimer into monomers and abolished DBDS binding (Boodho & Reithmeier, 1984).

A large body of information has been assembled on the mechanisms of denaturation of water-soluble proteins (*see* Tanford, 1968; 1970), but much less is

[1] *Abbreviations:* SDS, Na dodecyl sulfate; DBDS, 4,4'-dibenzamidostilbene disulfonate; NEM, N-methyl maleimide; *p*CMBS, *p*-chloromercuribenzene sulfonate; DMSO, dimethylsulfoxide; TU, thiourea; DMU, dimethylurea.

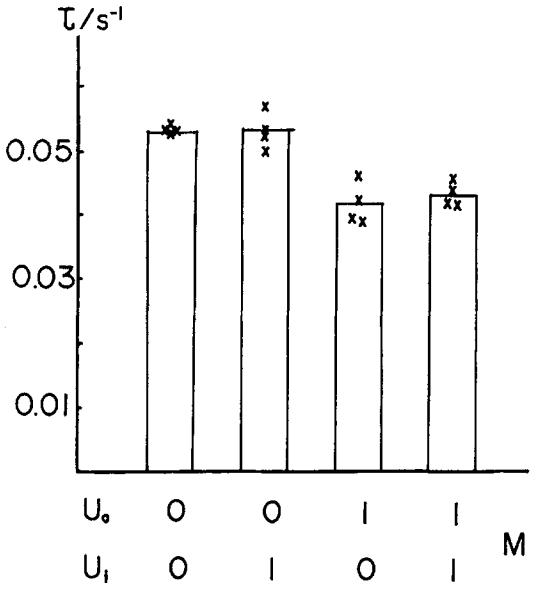

Fig. 1. Effect of urea on Cl equilibrium exchange. Cells were preincubated either in the presence or absence of 1 M urea and tracer Cl efflux was measured into media with or without 1 M urea. The rate of Cl transport depends only on the (initial) extracellular urea concentration since urea can rapidly equilibrate across the membrane and the small intracellular compartment adjusts its urea concentration to that of the extracellular compartment

known about membrane-bound proteins. The purpose of this study is to use band 3 as a model protein to study the behavior of membrane-embedded proteins under denaturating conditions. We used urea since it is very permeant (Brahm, 1983; Mayrand & Levitt, 1983); it equilibrates rapidly (within seconds or less) across the membrane thus alleviating concerns about possible osmotic side effects during the anion flux experiments. Another reason for examining urea's effect on band 3-mediated anion exchange is physiological. On their way through the microcirculation of the kidney, erythrocytes are exposed to fairly high urea concentrations: up to 600 mM in the human and even more in the rat kidney (Macey, 1984). It would therefore be interesting to know whether the red cells' function in removing CO_2 via Cl/HCO_3 exchange are impaired to any significant extent.

Our studies show that urea has an inhibitory effect on red cell chloride exchange, but with a relatively low affinity of about 0.4 M^{-1}. Its alkyl derivatives, however, possess significantly higher potencies. The data suggest that this inhibition is most likely due to nonspecific denaturation and not due to a physical and kinetic link between urea and anion transport as proposed by Solomon and co-workers (1983). Possibly the most significant observation is that the inactivation of Cl exchange does not exhibit the strongly sigmoidal behavior observed in a large number of water-soluble proteins for a transition from the native to a denaturated state. This work has been previously reported in preliminary form (Fröhlich & Jones, 1985).

Materials and Methods

Media

The solutions used for washing and preincubating the cells and for the tracer flux experiments were prepared from two types of stock solutions: Cl-stock, 150 mM KCl, 27 mM glycylglycine; and citrate-stock, 25 mM K-citrate, 200 mM sucrose, 27 mM glycylglycine, all of pH 7.6 (at room temperature). In addition they contained the required concentrations of urea or its analogs. The two types of stock media were mixed to obtain the desired Cl concentrations (Gunn & Fröhlich, 1979). For the experiments in Fig. 6, the efflux media were modified to contain the following: a) 20 mM KCl, 280 mM thiourea, b) 20 mM KCl, 250 mM sucrose, c) 20 mM KCl, 250 mM sucrose, 280 mM thiourea, or d) 20 mM KCl, 480 mM sucrose; all were buffered by 10 mM glycylglycine at pH 7.6. Solution a) caused lysis within several minutes at room temperature, but the entry of thiourea was sufficiently slow at the experimental temperature of 0°C so that the cell volume did not change appreciably during the experiment and tracer Cl efflux could easily be measured.

Urea and thiourea were obtained from Sigma, and methylurea, dimethylurea, propylurea and butylurea were obtained from Eastman. In control experiments to test for possible inhibitory effects by cyanate contaminations, we used urea and dimethylurea recrystallized from ethanol. For this these compounds were dissolved to saturation in warm ethanol. The saturated solution was slowly cooled down, first to room temperature and then in the refrigerator. The crystals were filtered in a Buechner funnel and dried in a stream of air. Removal of residual solvent was achieved by storing the crystals under vacuum in a desiccator.

Cell Preparation

Fresh blood (O.F. and S.C.J.) was drawn for each experimental series into heparin-containing centrifuge tubes. Plasma and white cells were removed by centrifugation and aspiration, and the cells were washed repeatedly in urea-free Cl-stock by repeatedly resuspending and centrifuging. For the experiments with the urea analogs, which permeate more slowly than urea, the cells were divided into aliquots and separately pre-equilibrated with Cl-stock solutions containing the desired alkylurea concentrations. After several washes near room temperature to achieve equilibration of the intracellular concentrations, the cells were resuspended to a hematocrit of about 40%, put back onto ice, and ^{36}Cl (as NaCl from ICN) was added. After another equilibration period (5 min) the suspension was transferred into nylon tubes for packing by centrifugation (Gunn & Fröhlich, 1979).

Flux Experiments

The experimental flux procedure was virtually the same as the standard procedure used previously (Gunn & Fröhlich, 1979).

The packed cells were injected into 100 volumes of well-stirred and thermostatted (0°C) efflux media to start tracer efflux. Five samples were withdrawn in 4- to 5-sec intervals into a collecting syringe through two fiber glass filters (MFS) contained in Millipore filter holders. From the time course of the appearance of tracer Cl in the filtrates the efflux rate constant (k, in units of sec^{-1}) was determined. In some instances we calculated the Cl flux rate [J_{Cl}, in units of mmol (kg cell solids · min)$^{-1}$] from k and the cellular Cl content (Gunn & Fröhlich, 1979). However, this is generally not necessary since the water content (and thus Cl content and concentration) of the cells did not change significantly with urea loading except at the highest concentrations where urea contributed to the dry cell mass. In several experiments the data are therefore given as rate constants since they all are proportional to the flux by the same scaling factor. Some of the experiments differ quantitatively from each other in the measured rate constants or flux rates under comparable conditions. These differences arose since we used the blood of two different donors which intrinsically differ in their maximal Cl flux rates, presumably due to differences in the number of band 3 molecules per cell (Fröhlich & Gunn, 1981).

Results

The rate of Cl exchange in the presence of 1 M (both intracellular and extracellular) urea is 20 to 25% slower than in the absence of urea (first and fourth bar in Fig. 1). This inhibition occurs very rapidly: the time course of tracer efflux follows very closely an exponential time course over the experimental period between 3 and 20 sec after mixing of the packed cells with the efflux medium. Also, the extrapolated zero-time intercept is the same for both experimental conditions (*not shown*). This means that inhibition by urea occurs within a very brief time after mixing (less than 2 sec). It is remarkable that the time course of the onset of inhibition is equally unresolvable when tracer Cl efflux is measured from urea-free cells into urea-containing efflux medium (third column of Fig. 1). This may be explained by the very high permeability for urea which permits equilibration of any initial concentration gradient within seconds or less (Brahm, 1983; Mayrand & Levitt, 1983). Rapid equilibration also explains the observation that the extent of inhibition is independent of the initial intracellular urea concentration (*compare* columns 1 with 2, and 3 with 4) since the relatively small intracellular compartment adjusts its urea concentration to that of the large extracellular compartment. Indeed, the equilibration of urea occurs so rapidly that tracer Cl efflux can be measured from cells that were preloaded with 1 M urea (column 2 of Fig. 1) into urea-free saline without causing measurable hemolysis. Again one observes strict exponential behavior of tracer efflux (*not shown*) which indicates that the inhibition by urea is just as rapidly reversed as it is induced.

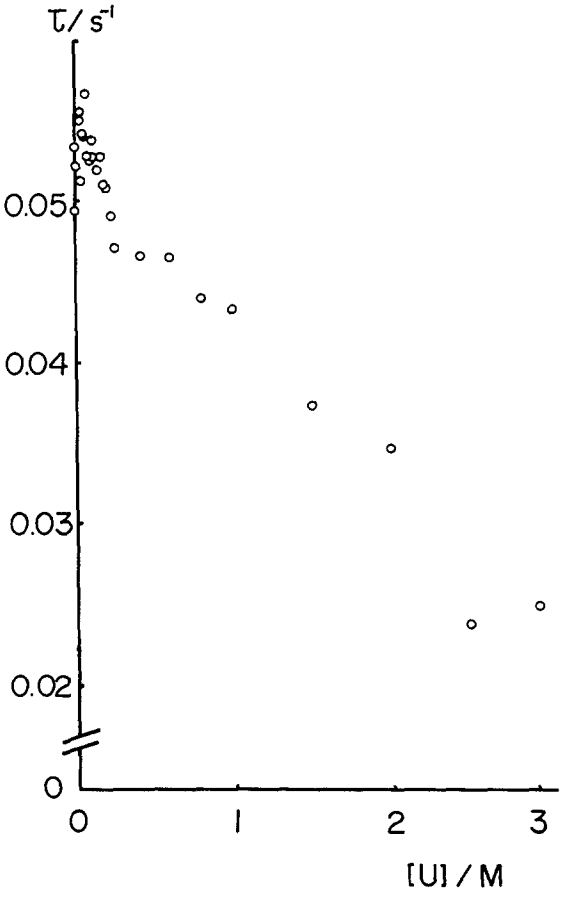

Fig. 2. Concentration dependence of Cl transport inhibition by urea. For these experiments the tracer-loaded, urea-free cells were injected into Cl-media containing different urea concentrations. It is assumed that the intracellular urea concentration essentially reached equilibrium with the extracellular concentration before the first time point of efflux was collected

Figure 2 shows the urea concentration dependence of Cl exchange. These experiments were performed by injecting urea-free, tracer-loaded cells into media of the indicated urea concentrations, relying on the rapid equilibration of intracellular with extracellular urea concentrations before the first experimental time point. The data show a strong inhibition of Cl transport as the urea concentration is raised to 3 M, with half-maximal inhibition occurring around 2.5 M. It was not possible to examine higher urea concentrations since above 2.5 M a significant fraction of the cells lysed.

We also examined the inhibitory effect of several alkyl derivatives of urea as well as of thiourea since these compounds had been shown with other proteins to possess a higher denaturating potency than urea (Elbaum et al., 1974; Warren & Gordon, 1976). This affinity series also applies to the anion transporter (Figs. 3 and 4). Since these urea analogs

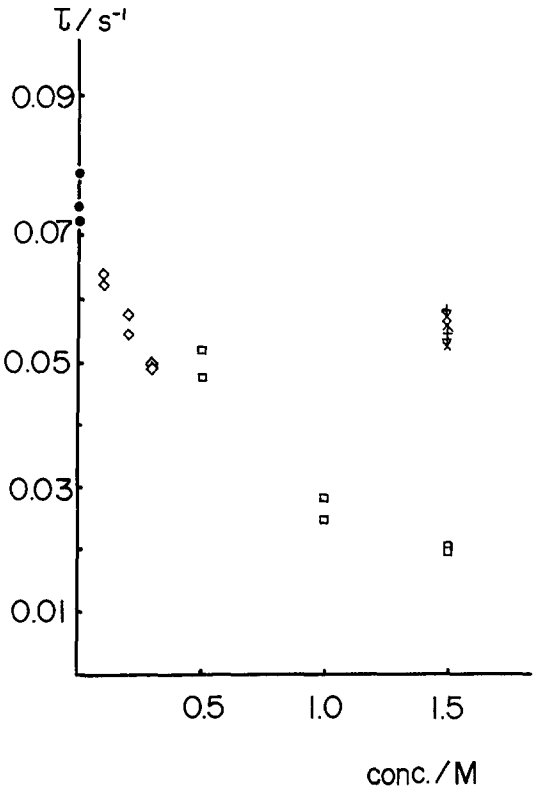

Fig. 3. Inhibition of Cl exchange by dimethylurea and thiourea. For these experiments the cells were pre-equilibrated with Cl-media containing the appropriate concentrations of dimethylurea or thiourea and Cl exchange was measured into the same media. ●: control fluxes without inhibitor; ◇: thiourea; □: (recrystallized) dimethylurea; x: urea as purchased; +: twice recrystallized urea

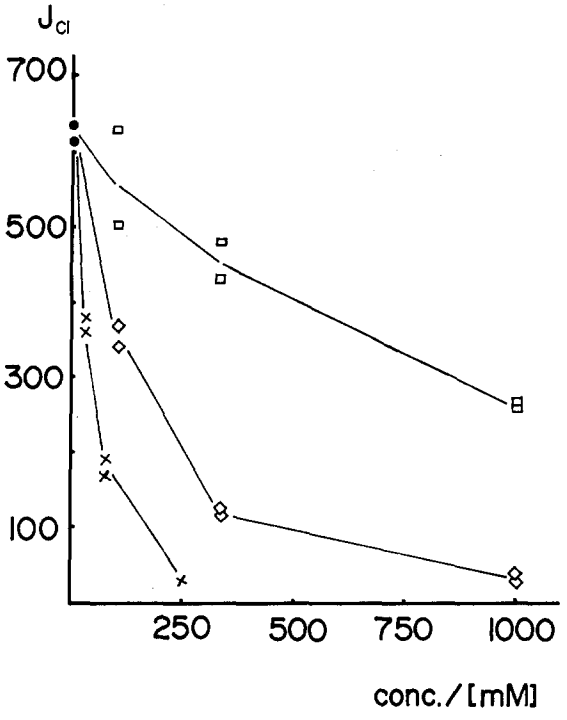

Fig. 4. Inhibition of Cl exchange by methylurea (□), propylurea (◇) and butylurea (×), from pre-equilibrated cells; (●), control flux. The experimental details are the same as in Fig. 3. The units of the chloride exchange rate are mmol (kg cell solids · min)$^{-1}$

permeate the membrane more slowly than urea, we preequilibrated the red cells with the intended concentrations of inhibitor. Figure 3 indicates that the inhibitory potencies of dimethylurea and thiourea are in the range of 500 mM. (For unknown reasons, the flux values at 500 mM DMU are higher than expected; when these two points are omitted, the Dixon plots of the data for both TU and DMU are nearly the same with an inhibitory constant of 520 mM.) In the data of Fig. 4, half-inhibition of Cl exchange was achieved by methylurea, propylurea and butylurea at 830, 150 and 60 mM, respectively. This series is similar to that found with water-soluble proteins (Elbaum et al., 1974; Warren & Gordon, 1976).

Figure 3 also presents the results of control experiments in which we examined whether the inhibition of Cl transport was exclusively due to urea or whether it could also stem from contaminants which are produced during storage by oxydation or hydrolysis. One possible anionic contaminant, cyanate, could bind to the anion transporter as competitive inhibitor and could be present in sufficient amounts at the high urea concentrations used. However, we found that doubly recrystallized urea had the same inhibitory effect as urea that was used directly from the storage bottle. This indicates that the inhibitory effect is due to urea and not due to contaminations.

It is noteworthy that none of the curves in Figs. 2–4 exhibits a strongly sigmoidal concentration dependence as is often observed with urea-induced denaturation of proteins. Some might even give the appearance of simple hyperbolic inhibition (Segel, 1975), but regraphing the data of Fig. 4 as Dixon plots reveals that the plots are nonlinear and bend upward (*not shown*). We examined this observation in more detail in order to learn to which extent this inhibition occurs by hyperbolic kinetics (which implies that only one urea molecule binds and causes inhibition). For this we chose dimethylurea (DMU) since it has a higher inhibitory potency than urea. The results of flux studies at different dimethylurea concentrations and at different extracellular chloride concentrations are plotted as Dixon plots in Fig. 5. The data points do not lie on a straight line. This is best explained by assuming that more than one dimethylurea molecule can bind and inhibit at a time, which is expected for this type of denaturant.

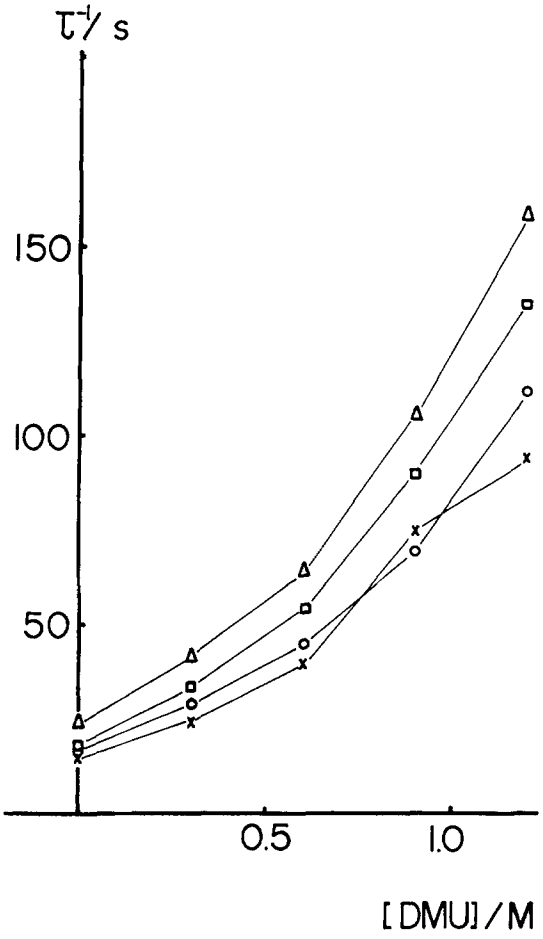

Fig. 5. Dixon analysis of Cl exchange inhibition by dimethylurea (DMU) from preincubated cells. The data are plotted as inverse of the rate constant *vs.* the inhibitor (DMU) concentration, at different substrate (Cl_o) concentrations: Cl_o was 16 (×), 8 (○), 4 (□) and 2 mM (△), respectively

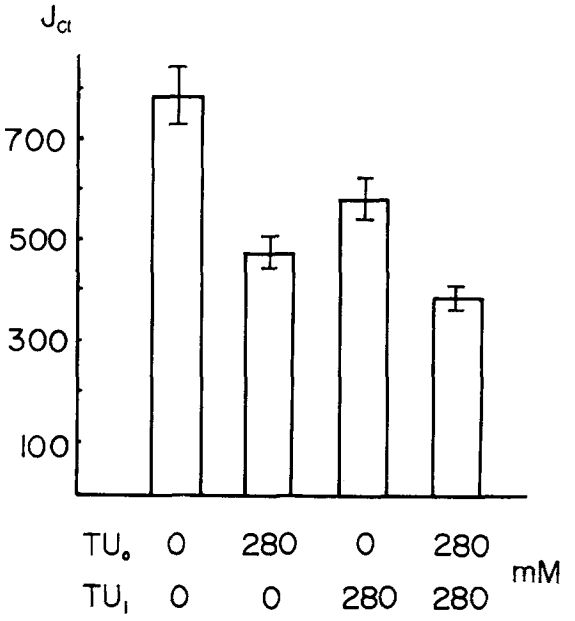

Fig. 6. Sidedness of Cl transport inhibition by thiourea (TU). Two types of cells were prepared which differed in their TU content, by incubation in TU-free Cl-medium or in Cl-medium plus 280 mM TU (to achieve TU_i = 280 mM). The first cell type was used with buffered (10 mM glycylglycine) efflux media either containing 20 mM KCl and an isotonic balance of 250 mM sucrose (first column), or 20 mM KCl, 280 mM TU (second column). The second cell type was used with efflux media containing either 20 mM KCl, 480 mM sucrose (third column) or 20 mM KCl, 280 mM TU, 250 mM sucrose (fourth column). The units of the chloride exchange rate are mmol (kg cell solids · min)$^{-1}$. The bars represent averages of 8 to 10 flux experiments and their standard deviations

The Hill coefficient for these data (*not shown*) yielded 1.5 to 1.7, which is the minimal number of inhibitory dimethylurea molecules per band 3 molecule. When only the fluxes at 0 and 300 mM DMU are used where one might suppose a closer adherence to a linear dependence, the data obtained at 8, 4 and 2 mM Cl_o intersect very close to the abscissa at −370 mM. The data at Cl_o = 16 mM give an *x*-intercept of −600 mM, probably because the flux at zero DMU was inappropriately low. The equivalent Cornish-Bowden plot of $[Cl_o]/k$ *vs.* [DMU] (Segel, 1975) yielded lines that intersect on the abscissa near −410 mM (*not shown*). All this is consistent with the notion that DMU and probably also the other urea derivatives are noncompetitive inhibitors that do not interfere with the binding of transported chloride.

The relatively slower permeability of some of the urea analogs compared to that of Cl transport permitted us to examine an aspect of urea binding that is not possible with isotropic water-soluble proteins: it is possible to expose selectively the extracellular or the intracellular aspects of the anion transport protein to the denaturant. Experiments of this type are shown in Fig. 6 for thiourea whose time course of equilibration at 0°C is in the order of several minutes (Wieth et al., 1974; Fröhlich & Trammel, 1987). The data suggest that extracellular thiourea has a higher potency than intracellular thiourea. Also, the effects of intracellular and extracellular thiourea, although not strictly additive, are compounded when they are present simultaneously.

In the final experimental series we examined the effect of urea on another kinetic aspect of anion exchange, namely self-inhibition. Self-inhibition means that in the high concentration range the rate of anion transport decreases with increasing substrate concentrations. Whether this effect is due to binding of a nontransported anion to a "modifier site" (Dalmark, 1976), the binding of a second

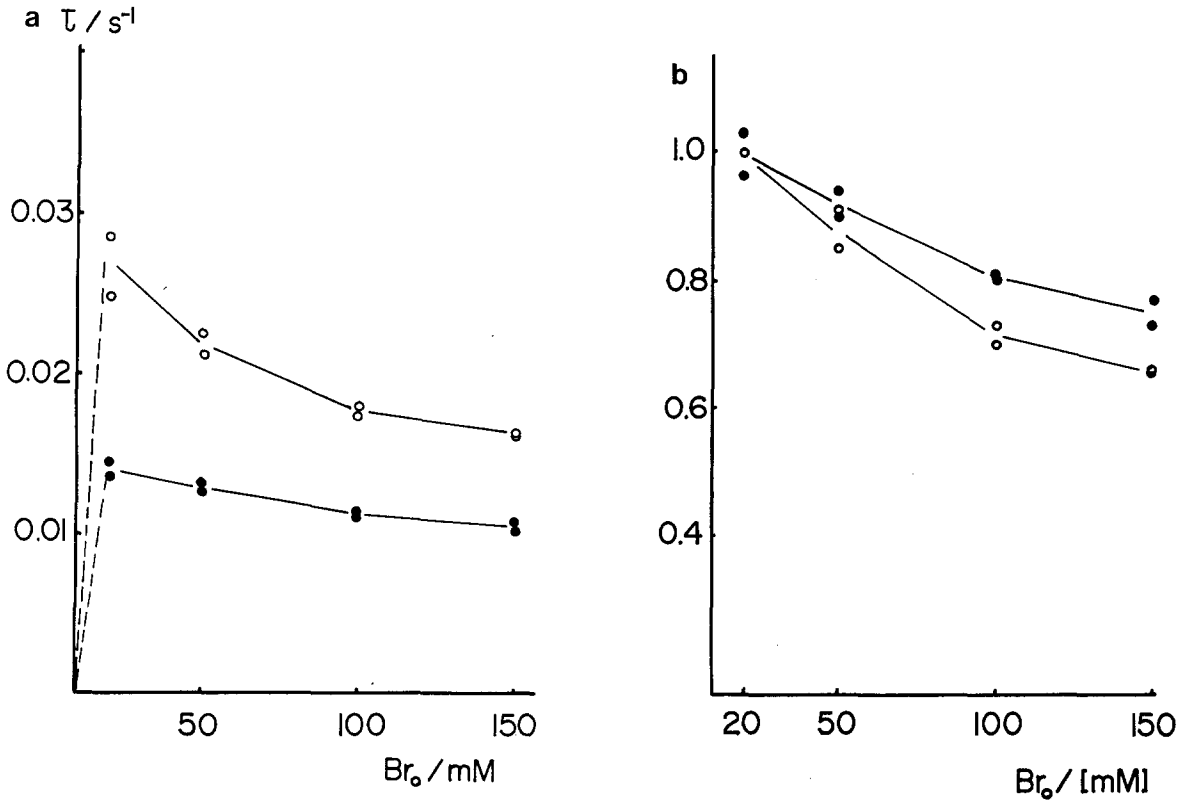

Fig. 7. Effect of urea on anion exchange self-inhibition. Tracer Cl efflux was measured into Br-containing media in which bromide was partially substituted by citrate/sucrose, in the absence (○) or presence (●) of 2 M urea. (*a*) Rate constant of Cl/Br exchange as function of Br_o; the stimulation of exchange by low Br_o to its maximum at 20 mM is indicated by the dashed line. (*b*) Replot of the same data, normalized by the values at 20 mM (at zero urea, only the lower of the two data points was used for normalization, in order to demonstrate the minimal difference between the two conditions)

transportable anion to the transport mechanism (Salhaney & Rauhenbuehler, 1983; Tanford, 1985), or due to an interaction among two band 3 monomers, is not yet known. Urea can dissociate the protein dimer (Boodhoo & Reithmeier, 1984), so that it might be useful to examine whether it has an effect on self-inhibition. Since self-inhibition is more prominent with Br than with Cl, we tested the effect of urea on Cl_i/Br_o exchange at different concentrations of Br_o (Fig. 7*a*). As expected, 2 M urea inhibited Cl_i/Br_o exchange by approximately 50% at Br_o = 20 mM, but its inhibitory effect was less pronounced at higher Br_o values where self-inhibition was stronger. This is more clearly visible in Fig. 7(*b*) where we normalized the fluxes to the maximal exchange rate at Br_o = 20 mM.

Discussion

In this study we have measured the effect of urea and some analogs on the rate of erythrocyte anion exchange. The purpose of these experiments was threefold: 1) to examine the hypothesis that urea and chloride transport may occur by the same transport mechanism; 2) to examine whether the urea concentrations to which erythrocytes are exposed in the circulation of the kidney have an adverse effect on anion transport (physiologically, Cl/HCO_3 exchange); and 3) to learn whether and how a membrane-intrinsic protein can be influenced by a classical denaturating agent, urea.

The first hypothesis was stated by Solomon and co-workers (1983) who sought an explanation for the high urea permeability of the erythrocyte membrane and for the lack of a membrane protein with a sufficient abundance (besides the anion and the glucose transporter). There is considerable evidence that suggests that anions and urea are not transported along the same transport pathway (*see* Macey, 1984) although it cannot be excluded that both pathways may exist in different domains of the same (band 3) protein. We have demonstrated that urea transport is influenced neither by the conformational state nor by the turnover rate of the anion transport mechanism (Jones & Fröhlich, 1984), and

Galey and Brahm (1984) have reported similar results. This also argues against the hypothesis of a common mechanism. The data presented here show that, in the inverse direction, urea does have an effect on anion exchange. However, they also suggest that this effect is not due to a direct kinetic link between urea and chloride transport but rather that it is due to gradual inactivation of the anion transport protein. The major argument against a kinetic link comes from a comparison of the inhibitory potencies for urea and thiourea with the apparent transport affinities of these compounds. Figures 1 and 2 show half-inactivation concentrations of 2.5 and 0.5 M for urea and thiourea, respectively. Both values are much higher than the respective $K_{1/2}$ values of 200 to 300 and 15 to 20 mM (Brahm, 1983; Mayrand & Levitt, 1983). Also, the type of inhibition of Cl exchange by dimethylurea is mixed if not strictly noncompetitive which would not be expected if urea were somehow blocking the chloride transport pathway. Finally, the nonlinear Dixon plots of Fig. 5 and similar plots of the other data (*not shown*) indicate that more than one dimethylurea or urea molecule can bind to band 3 and inhibit anion transport. The urea flux experiments by Brahm (1973) and Mayrand and Levitt (1983), on the other hand, revealed simple Michaelis-Menten kinetics which are best explained by the binding of a single urea molecule to the urea transport mechanism.

Dorogi and Solomon (1985) have studied the effect of thiourea on the stilbene-binding properties of ghosts that had been pretreated with NEM and *p*CMBS. These experiments revealed a modulation of the binding kinetics of the fluorescent stilbene analog DBDS by thiourea, with thiourea binding constants in the range of 10 μM to 0.4 mM. The authors inferred from these data support for a physical relationship between the urea and the anion transport mechanisms, that is for the involvement of a single protein or protein complex. However, the origin of these high affinity effects is not clear since they are up to three orders of magnitude higher than the apparent transport affinities reported by Brahm (1983) and Mayrand and Levitt (1983). In the discussion above we argue that the inhibitory effect on band 3 transport function occurs at a concentration that is another order of magnitude larger. Again, this does not support the notion of a specific kinetic relationship between urea and anion transport. It is, however, possible that increasing the concentration of a denaturant such as urea leads to an increased number of bound denaturant molecules with decreasing affinities which could gradually affect different aspects of transport (*see below*).

With respect to our second purpose we can conclude that anion transport of the red cells might indeed be inhibited as they pass through the inner medulla of the kidney. However, this inhibition should be rather limited at 600 mM urea (10 to 15%) and should be of no significant physiological consequence. As an aside, it is interesting to note, however, that the high urea permeability serves as a physiological protection mechanism for the red blood cell since it helps avoid a possible volume overshoot as they leave the zone of high osmolality in the renal medulla (Macey, 1984).

Since it appears reasonable to conclude that the urea effect on chloride exchange is most probably not due to specific transport site interactions between chloride and urea, one can examine in more detail the suggestion that urea and its analogs act nonspecifically by altering the conformational state of the transport protein. The mechanism of inactivation by denaturation of water-soluble proteins has been subject to a great number of studies on many different proteins and different denaturants (*see* Tanford, 1968; 1970; Ghélis & Yon, 1982). Thermodynamic analyses indicate that the effect of urea on the protein conformational state is largely through localized interactions with hydrophobic side chains and through hydrogen bond formation with peptide groups (Tanford, 1970). Translating this thermodynamic argument into a more physical understanding, particularly in terms of urea binding sites, is difficult. Other factors probably have to be considered as well. There are differences between the action of the guanidinium ion, a charged urea analog, the neutral urea, and the still less polar alkyl ureas. For example, at concentrations that do not cause complete unfolding of the protein, guanidine appears to affect only regions on the water-exposed surface of chymotrypsin whereas urea caused rearrangements of residues on the surface as well as the hydrophobic interior of the protein (Hibbard & Tulinsky, 1978). This difference is most probably due to the charge difference which at these subdenaturing concentrations permits urea but not guanidine to penetrate into the protein. Differences have also been observed between urea and its more nonpolar alkyl derivatives (Warren & Gordon, 1976). Urea rearranges chymotrypsin into a nonnative but still regularly ordered conformation, which is similar to the effect of short-chain alcohols. Alkyl ureas, on the other hand, disrupt this protein more thoroughly, transforming it into a random-coil state.

Relatively little is known about the secondary and tertiary structure of band 3 within the bilayer, except for the significant alpha-helical content (58%) of the carboxy-terminal, membrane-associated domain (Oikawa, Lieberman & Reithmeier,

1985). One might expect that the surrounding lipids provide protection for the protein against polar or charged denaturants but possibly might be detrimental in the case of nonpolar denaturants. Indeed, guanidine has been found to affect the different segments of band 3 to a different extent: the cytoplasmic domain of band 3 is completely converted to a random-coil state by 3 M guanidine whereas the membrane-resident segment is much more resistant (Oikawa et al., 1985). The increased potencies of the alkylureas with increasing chain length could be explained by their increased lipid solubility which enables them to partition more easily into the hydrophobic protein (and membrane) interior. This would explain the same potency sequences for band 3-mediated anion exchange as for the conformational states of chymotrypsin (Warren & Gordon, 1976) or hemoglobin (Elbaum et al., 1974). In a study of the inhibitory effects of alkanols on red cell sulfate transport, Forman and co-workers (1985) correlated the inhibitory potency of these agents with their lipid solubility. They found that the effectiveness of these alcohols, when calculated as concentration in the membrane interior, was the same for ethanol through heptanol. It is quite possible that a similar analysis of the urea derivatives studied here could yield quantitatively similar inhibitory constants for the different membrane-dissolved ureas. Such a similarity would suggest that the interactions that lead to the inactivation of the anion transport protein are the same for all ureas. A possible mechanism would be the disruption of hydrogen bonds within the membrane-spanning alpha-helices although one has to keep in mind that the alkyl-substituted ureas possess a lesser ability to form hydrogen bonds than urea.

An interesting observation is that the inhibition of chloride exchange occurs rather gradually with increasing urea concentrations. This is similar to the inhibitory effect of the alkanols (Forman et al., 1985) and of glycerol and DMSO (Gunn & Kirk, 1976) on anion transport but unlike the strongly sigmoidal dependence that is observed with some other proteins. In fact, it takes a Dixon analysis such as in Fig. 5 to demonstrate the deviation from hyperbolic behavior. Our calculated Hill coefficient of 1.5 to 1.7 is slightly higher than that of 1.2 to 1.4 observed by Forman et al. (1985) for the alkanols and certainly higher than unity. This excludes the binding of a single urea molecule as inhibitor. However, the measured Hill coefficient is still rather low compared to values of around 10 or more for some water-soluble proteins (*see*, for example, Tanford, 1970; Ghélis & Yon, 1982). Most likely the Hill coefficient represents an underestimate of the number of inactivating urea molecules. Such a discrepancy could arise if not all urea binding sites possess the same affinity, and if with gradual loosening of the tertiary structure more, lower-affinity sites become accessible. The asymmetric inhibition of Cl exchange by extracellular and intracellular thiourea can be considered evidence in favor of different affinities for different denaturant binding sites.

There are other possible explanations for the low apparent cooperativity: For example, the inactivation of the anion transporter may not follow a simple two-state transition scheme between native and denaturated, or the structural indicators such as circular dichroism or the fluorescence of aromatic amino acids that are frequently used to monitor denaturation, may be much less sensitive to subtle changes in structure than indicators of enzymatic activity. The first mechanism is nearly equivalent to the previous explanation that gradually more urea binding sites are exposed. In the extreme case of many intermediate states, the transition to the denatured state would then be nearly continuous. However, a continuous mechanism applies very rarely, if at all. The second explanation is also attractive in the light of findings that some proteins lose their enzymatic at urea or guanidine concentrations that are much below those necessary for detectable changes in structural parameters (Yao, Tian & Tsou, 1984; Strambini & Gonelli, 1986). The observed rapid reversibility of the urea inhibition (albeit from a relatively low degree of inhibition) is also consistent with the notion of limited structural changes of the transport mechanism at these urea concentrations. However, a firmer answer to this question awaits a more detailed comparison of functional parameters (transport or inhibitor binding) with conformational state indicators such as circular dichroism or intrinsic fluorescence).

The mechanism of the urea effect on transport self-inhibition is at the present time a matter of speculation. Urea and guanidine denature the band 3 protein to the extent that it cannot bind stilbene inhibitors (Boodhoo & Reithmeier, 1984). At the same time, they dissociate the band 3 dimer (or possibly tetramer) complex into its component monomers. Although most of the experimental evidence points to the individual band 3 peptide as an independently functioning unit (*see* Jennings, 1984), the dimeric state appears to be important for proper transport function, possibly by stabilizing the tertiary structure of the component monomers. One could therefore suppose that the dissociation of the dimer is an additional factor that leads to transport inactivation, independent of a disruptive effect of urea on intramolecular alpha-helical hydrogen bonding. It is more difficult to account for the reduced inhibition by urea at high anion concentra-

tions. Either one has to postulate that at high anion concentrations monomer-monomer interactions occur as has been postulated in some instances (Salhaney & Rauhenbuehler, 1983; Verkman, Dix & Solomon, 1983), or that anion binding stabilizes the structure of the anion transport mechanism against denaturation by urea. It is known, for example, that covalent stilbene binding stabilizes the membrane-intrinsic segment of band 3 against thermal denaturation (Oikawa et al., 1985). However, dimethylurea appears to be a noncompetitive inhibitor of anion exchange, which implies that there are no interactions between the binding of denaturating urea and the binding of the transported chloride. This would exclude transported anions from stabilizing the protein structure and point towards binding of the self-inhibitory anion as the stabilizing factor.

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