Factors affecting counteraction by methylamines of urea effects on aldose reductase

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ABSTRACT The concentration of urea in renal medullary cells is high enough to affect enzymes seriously by reducing \( V_{\text{max}} \) or raising \( K_m \) yet the cells survive and function. The usual explanation is that the methylamines found in the renal medulla, namely glycero- phosphocholine and betaine, have actions opposite to those of urea and thus counteract its effects. However, urea and methylamines have the similar (not counteracting) effects of reducing both the \( K_m \) and \( V_{\text{max}} \) of aldose reductase (EC 1.1.1.21), an enzyme whose function is important in renal medullas. Therefore, we examined factors that might determine whether counteraction occurs, namely different combinations of assay conditions (pH and salt concentration), methylamines (glycero- phosphocholine, betaine, and trimethylamine \( N \)-oxide), substrates (DL-glyceraldehyde and D-xylose), and a mutation in recombinant aldose reductase protein (C298A). We find that \( V_{\text{max}} \) of both wild-type and C298A mutant generally is reduced by urea and/or the methylamines. However, the effects on \( K_m \) are much more complex, varying widely with the combination of conditions. At one extreme, we find a reduction of \( K_m \) of wild-type enzyme by urea and/or methylamines that is partially additive, whereas at the other extreme we find that urea raises \( K_m \) for D-xylose of the C298A mutant, betaine lowers the \( K_m \) and the two counteract in a classical fashion so that at a 2:1 molar ratio of betaine to urea there is no net effect. We conclude that counteraction of urea effects on enzymes by methylamines can depend on ion concentration, pH, the specific methylamine and substrate, and identity of even a single amino acid in the enzyme.

High concentrations of urea are present in the tissues of marine elasmobranchs and in the mammalian renal medulla. Urea generally destabilizes biological macromolecules, altering their structure and function. Such effects are expected to be deleterious. However, the urea-rich tissues also contain high concentrations of certain methylamine compounds, principally trimethylamine \( N \)-oxide (TMAO) in elasmobranchs (1) and glycine betaine (betaine) and glycero- phosphocholine (GPC) in mammalian renal medulla (2). These methylamines are believed to protect the cells from urea by stabilizing macromolecules and thus counteracting the actions of urea. The two effects are independently additive (1, 3). When the ratio of methylamines to urea is appropriate (often 1:2), their opposing effects are reported to counteract, preserving macromolecular structure and function. The theory of counteracting osmolites is strongly supported by the occurrence of methylamines in organisms and tissues containing high concentrations of urea (4), by survival in tissue culture of cells containing high levels of both urea and betaine, compared with the poor survival of cells containing only one or the other (5), and by many observations on isolated macromolecules in vitro (4).

However, we recently found that three different methylamines, namely TMAO, betaine, and GPC, do not counteract inhibition by urea of the enzyme aldose reductase (EC 1.1.1.21) (6). In fact, the methylamines all substantially decrease \( V_{\text{max}} \) of aldose reductase, similar to urea, and the effects of urea and the methylamines are partially additive rather than counteracting. This is especially surprising because aldose reductase normally functions in the presence of high urea concentration in the renal medulla. The enzyme catalyzes the production of large amounts of sorbitol from glucose, and the sorbitol protects renal medullary cells from the high concentration of salt that normally also is present. Thus, this enzyme, which has an important function in the renal medulla, is not protected from urea by methylamines, but rather is inhibited by both urea and the methylamines.

In addition to decreasing \( V_{\text{max}} \) of aldose reductase, urea also decreases its \( K_m \), measured with DL-glyceraldehyde as substrate. This result is also surprising because urea generally increases enzyme \( K_m \) (7), and we are unaware of any enzyme besides aldose reductase whose \( K_m \) is reduced by urea. The methylamines have an effect on \( K_m \) similar to that of urea. Thus, TMAO, betaine, and GPC all decrease the \( K_m \) of aldose reductase for DL-glyceraldehyde (6).

The purpose of the present studies was to examine conditions under which counteraction might occur between the effects of urea and methylamines on aldose reductase in order better to define the possible determinants of counteraction. To this end we tested both recombinant wild-type aldose reductase and its C298A mutant, and we varied the substrate used as well as the conditions of the assay.

MATERIALS AND METHODS

Preparation of Recombinant Aldose Reductases. Wild-type (8) and C298A (9) mutant human aldose reductase were overexpressed in Escherichia coli and purified as previously described (8). The enzymes, stored in running buffer (5 mM sodium phosphate, pH 7.4/7 mM 2-mercaptoethanol/0.1 mM EDTA) at 4°C for no more than one month, were diluted to working concentrations with the same buffer immediately before each assay.

Measurement of Aldose Reductase Activity. Quartz cuvettes containing reaction mixture (complete, except for the substrate, which was either DL-glyceraldehyde (Sigma G 5001) or D-xylose (Sigma X 1500)) were prewarmed for 2 min to 37°C in the temperature-controlled six-cell positioner (model CPS-240A) of a Shimadzu UV-1601 recording spectrophotometer. Then, the substrate, contained in 10% of the final volume, was added with mixing, and oxidation of NADPH was followed at 340 nm with a Shimadzu UV-1601 recording spectrophotometer.

Abbreviations: TMAO, trimethylamine \( N \)-oxide; GPC, glycero- phosphocholine.

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RESULTS

Both Urea and Methylamines Inhibit Human Recombinant Aldose Reductase. Using DL-glyceraldehyde as substrate, we previously found that a high concentration of urea or betaine inhibits aldose reductase activity in homogenates of renal medullary epithelial cells (PAP-HT25) (12) and that urea, betaine, TMAO, or GPC inhibits the activity of recombinant rat aldose reductase (6). In the latter study no counteraction between urea and the methylvamines was apparent. This finding is confirmed in the present study of recombinant human aldose reductase (Table 1). When DL-glyceraldehyde is used as substrate, urea and the individual methylvamines each reduce \( V_{\text{max}} \) and the effects of combinations of urea and the methylvamines are partially additive. There is one apparent difference, however, between the earlier studies and the present ones. TMAO decreases \( V_{\text{max}} \) only by 8% (Table 1), which is much less than the 53% decrease previously observed (6). The results with D-xylose as substrate are similar to those with DL-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Urea (1.0 M)</th>
<th>Methylamine (0.5 M)</th>
<th>( V_{\text{max}} ) % of control</th>
<th>( K_m ) % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Glyceraldehyde</td>
<td>-</td>
<td>None (control)</td>
<td>100.0 (9)</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>None</td>
<td>43.4 ± 1.2 (9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TMAO</td>
<td>91.6 ± 0.5 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TMAO</td>
<td>39.1 ± 0.5 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Betaine</td>
<td>40.3 ± 1.2 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPC</td>
<td>27.3 ± 0.2 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPC</td>
<td>35.7 ± 0.1 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPC</td>
<td>21.4 ± 1.4 (3)</td>
<td></td>
</tr>
<tr>
<td>D-xylose</td>
<td>-</td>
<td>None</td>
<td>100.0 (9)</td>
<td></td>
</tr>
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<td>None</td>
<td>48.4 ± 1.0 (9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TMAO</td>
<td>98.0 ± 0.6 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TMAO</td>
<td>43.6 ± 0.6 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Betaine</td>
<td>39.6 ± 0.2 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPC</td>
<td>28.3 ± 0.6 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPC</td>
<td>35.5 ± 0.5 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPC</td>
<td>29.5 ± 0.5 (3)</td>
<td></td>
</tr>
</tbody>
</table>

*Controls contained no urea or cosolvent. All experimental values are significantly less than control (\( P < 0.05 \)), except for TMAO with D-xylose and no urea. Number of measurements is given in parentheses.

glyceraldehyde (Table 1), except that the effect of TMAO is even smaller and is not statistically significant. The following experiments were designed to investigate why effects of TMAO were smaller or absent in the present study compared with a substantial effect in the previous study (8).

Buffer Composition Affects the Action of TMAO on Aldose Reductase. We used D-xylose, rather than DL-glyceraldehyde, as substrate in most of the present studies because it had previously been used extensively with recombinant human aldose reductase (8) and offered the opportunity to test the generality of the previous findings with DL-glyceraldehyde. The strikingly different effects of TMAO led us to reexamine the conditions used in the two studies. In addition to the difference in enzyme preparations (recombinant rat versus human aldose reductase), the buffers also differ. Following the assays customary in different laboratories, 0.01 M potassium phosphate buffer, pH 6.0, had been used with the rat enzyme and 0.10 M sodium phosphate buffer, pH 7.0, was used in the present studies of the human enzyme.

When 0.01 M potassium phosphate buffer, pH 6.0, is used with human aldose reductase, 0.5 M TMAO inhibits \( V_{\text{max}} \) by 46% (Table 2), which is essentially the same result as previously with the rat enzyme (53% decrease) under the same conditions. In contrast, when 0.10 M sodium phosphate buffer, pH 7.0, is used, TMAO inhibits \( V_{\text{max}} \) by 81% (Table 2), which is significantly less than the 86% inhibition previously observed (6).

Table 2. Effect of buffer composition on kinetics of wild-type aldose reductase with DL-glyceraldehyde as substrate (\( n = 3 \))

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Urea (1.0 M)</th>
<th>TMAO (0.5 M)</th>
<th>( V_{\text{max}} ) % of control</th>
<th>( K_m ) % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 M KPO(_4), pH 6.0</td>
<td>-</td>
<td>-</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>26.3 ± 0.2</td>
<td>23.7 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>54.4 ± 0.3</td>
<td>29.2 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>19.8 ± 0.3</td>
<td>9.5 ± 0.3</td>
</tr>
<tr>
<td>0.01 M KPO(_4), pH 7.0</td>
<td>-</td>
<td>-</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>43.6 ± 0.6</td>
<td>45.5 ± 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>76.3 ± 0.8</td>
<td>58.1 ± 1.2</td>
</tr>
<tr>
<td>0.10 M NaPO(_4), pH 7.0</td>
<td>-</td>
<td>-</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>46.0 ± 1.7</td>
<td>52.7 ± 2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>91.6 ± 0.6</td>
<td>81.7 ± 5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>39.1 ± 0.5</td>
<td>40.4 ± 0.9</td>
</tr>
</tbody>
</table>

*All experimental values are significantly less (\( P < 0.05 \)) than the controls, which contained no urea or TMAO. Control values (no urea or TMAO) for \( K_m \) are a, 0.371 mM; b, 0.087 mM; and c, 0.141 mM.
controls contain no urea or cosolvent. All experimental values are
present study than previously. As already explained for
and Figs. 1-3). However, the effect of TMAO is smaller in the
observed that urea, betaine, TMAO, and GPC greatly reduce
the difference in the buffers used in the two studies is
attributable due to the difference between the buffers, not
between the enzymes. Both the salt used in the buffer and the
pH contribute to the effect. Increasing pH from 6.0 to 7.0
increases the $V_{max}$ in the presence of 0.5 M TMAO from 54% to
76% of control, and changing the salt from 0.01 M potassium
phosphate to 0.10 M sodium phosphate increases the $V_{max}$
further to 92% of control (Table 2). Changing the buffer
conditions evidently affects the results with TMAO more than
with the other methylamines, but it is not clear why this occurs
(Table 2). In any event, the various cosolvents tested (urea
alone, betaine or GPC alone, and combinations of betaine or
GPC plus urea) all evidently inhibit aldose reductase activity
regardless of the particular buffer and species of the recom-
binan enzyme (Table 1 and ref. 6).

Urea and Methylamines Reduce the $K_m$ of Human Aldose
Reductase for dL-Glyceraldehyde and D-Xylose. We previously
observed that urea, betaine, TMAO, and GPC greatly reduce the
$K_m$ of recombinant rat aldose reductase for dL-
glyceraldehyde (6). In the present studies we confirm this
finding, using recombinant human aldose reductase (Table 3
and Figs. 1-3). However, the effect of TMAO is smaller in the
present study than previously. As already explained for $V_{max}$,
the difference in the buffers used in the two studies is
responsible. TMAO reduces the $K_m$ of human aldose reductase
much more when the buffer is 0.01 M potassium phosphate,
pH 6.0, than when it is 0.10 M sodium phosphate, pH 7.0
(Table 2). On the other hand, the effects of urea, betaine, and
GPC are similar with the two buffers, as seen by comparing the
results in Table 3 to the previous ones (6).

The purpose of the remaining studies was further to examine
this effect on $K_m$ by testing the effect of the cosolvents on the
recombinant C298A mutant of human aldose reductase.

Urea and Methylamines Inhibit the Activity of the C298A
Mutant of Human Aldose Reductase. The C298A mutant of
human aldose reductase has a high enzyme activity. $V_{max}$ of
C298A is 8.7 times that of wild type (9). Nevertheless, the
effects of urea and the methylamines on enzyme activity are
qualitatively similar, comparing the wild type and mutant.
Thus, betaine, GPC, or urea alone, and betaine or GPC
combined with urea, all reduce $V_{max}$ of both C298A (Table 4)
and wild-type aldose reductase (Table 1). The relative degrees
of inhibition differ from condition to condition, but there is no
general trend. One notable difference, however, is that with
d-xylene as substrate TMAO decreases the $V_{max}$ of C298A, but
not of the wild type.

Effects of Urea and Methylamines on $K_m$ Differ Greatly
Between the C298A Mutant and Wild-Type Aldose Reductase.
The $K_m$ of the C298A mutant is considerably higher than that
of the wild type (9). In the present studies the mean values with
dL-glyceraldehyde as substrate are wild type, 0.157 ± 0.005
mM, and C298A, 1.03 ± 0.06 mM, and with d-xylene as
substrate the values are wild type, 8.1 ± 0.3 mM, and C298A,
218.3 ± 7.6 mM.

Table 3. $K_m$ of wild-type aldose reductase with dL-glyceraldehyde
as substrate

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0.157 ± 0.006 (9)</td>
</tr>
<tr>
<td>+ Urea</td>
<td>0.075 ± 0.002 (9)</td>
</tr>
<tr>
<td>+ TMAO</td>
<td>0.116 ± 0.001 (3)</td>
</tr>
<tr>
<td>+ Betaine</td>
<td>0.057 ± 0.002 (3)</td>
</tr>
<tr>
<td>+ GPC</td>
<td>0.049 ± 0.006 (3)</td>
</tr>
</tbody>
</table>

* Controls contain no urea or cosolvent. All experimental values are
significantly less than the controls that were run simultaneously with
them ($P < 0.05$).

![Fig. 1](image1.png)  
Fig. 1. Effect of urea and TMAO on $K_m$ of human aldose reductase for d-xylene ($n = 3$). * Significantly different from control ($P < 0.05$). Additional significant differences with wild-type are TMAO versus urea and TMAO versus urea + TMAO. With C298A mutant, all other differences are significant. Mean control values of $K_m$ are wild-type, 6.9 mM, and C298A, 228 mM.

![Fig. 2](image2.png)  
Fig. 2. Effect of urea and GPC on $K_m$ of human aldose reductase for d-xylene ($n = 3$). * Significantly different from control ($P < 0.05$). Additional significant differences with wild-type ($n = 3$) are urea versus betaine and urea versus urea + betaine. With C298A mutant ($n = 5$), all other differences are also significant. Mean control values of $K_m$ are wild-type, 9.4 mM, and C298A, 229 mM.

![Fig. 3](image3.png)  
Fig. 3. Effect of urea and GPC on $K_m$ of human aldose reductase for d-xylene ($n = 3$). * Significantly different from control ($P < 0.05$). Additional significant differences with the C298A mutant are urea versus GPC and GPC versus urea + GPC. Mean control values of $K_m$ are wild-type, 7.8 mM, and C298A, 191 mM.
Urea elevates the $K_m$ of many enzymes (1, 7). The reduction of the $K_m$ of wild-type aldose reductase by urea observed in the present and previous studies (6) is exceptional in this regard. However, with d-xylose as substrate, the effect of urea on $K_m$ of the C298A mutant aldose reductase resembles its effect on most other enzymes and is strikingly different from its effect on wild-type aldose reductase. Thus, with d-xylose as substrate, urea raises the $K_m$ of the C298A mutant, rather than lowering $K_m$ as with the wild-type enzyme (Figs. 1–3).

With DL-glyceraldehyde as substrate, urea has little (Fig. 4) to no (Fig. 5) effect on $K_m$ of the C298A mutant. In contrast, urea decreases the $K_m$ for DL-glyceraldehyde of the wild-type enzyme by approximately 50% (Figs. 4 and 5).

Betaine Counteracts Elevation by Urea of the $K_m$ for d-Xylose of the C298A Mutant. Betaine at 0.5 M decreases the $K_m$ for d-xylose of the C298A by approximately the same amount as 1.0 M urea increases the $K_m$ (Fig. 2). When the two are added simultaneously in a 1:2 molar ratio, their effects counteract, and the $K_m$ remains at the control level. Counteraction with approximately a 1:2 ratio of methylamine to urea is believed to be especially significant because a similar ratio is found in tissues exposed to high urea (1). The present result with aldose reductase is surprising because counteraction is not apparent for the wild-type enzyme, which normally functions in the renal medulla where the urea concentration is high, but counteraction is apparent for the unnatural mutant.

With other methylamines (TMAO in Fig. 1 and GPC in Fig. 3) and substrate (DL-glyceraldehyde in Figs. 4 and 5), the results also differ between the wild type and C298A mutant, but exact counteraction does not occur with the mutant. Thus, with d-xylose as substrate, TMAO partially counteracts the increase in $K_m$ caused by urea (Fig. 1), but GPC does not (Fig. 3). With DL-glyceraldehyde as substrate and wild-type enzyme, the decreases in $K_m$ caused by urea and GPC or betaine are partially additive, whereas this is not the case with the C298A mutant (Figs. 4 and 5)—i.e., with the C298A mutant the result with urea plus methylamine is intermediate between the result with either alone.

**DISCUSSION**

Why do urea and methylamines have similar and partially additive effects on $K_m$ of aldose reductase in contrast to their counteracting effects on $K_m$ of many other enzymes?

The importance of keeping the $K_m$ of each enzyme within a narrow range to maintain optimal rates and regulation of catalysis has been emphasized, and the significance of counteraction between urea and methylamines for conservation of $K_m$ values has been stressed in this regard (1). However, we found previously (6) and confirm here that urea or methylamines individually lower the $K_m$ of wild-type aldose reductase, and that their effects are not counteractive, but are partially additive. In an attempt to understand what is special about aldose reductase, we first briefly review the chemical basis of counteraction and then the catalytic mechanism of aldose reductase.

The mechanism of counteraction is clearest with respect to protein thermal denaturation (3). Urea decreases stability of RNase T1, as manifest by a decrease in the temperature ($T_m$) at which unfolding of the protein occurs. Urea enhances unfolding of proteins by binding strongly to the unfolded form. A methylamine, TMAO, has the opposite effect of increasing stability, as manifest by higher $T_m$ values. TMAO is preferentially excluded from the protein, enhancing hydration of the protein and promoting folding. The two actions are independent and additive, so that at a molar ratio of urea to TMAO of...
approximately 2:1 there is no net effect—i.e., there is counteraction.

Thus, cosolvents such as methyamines and urea alter protein folding by affecting hydration of the exposed surface of the protein. Methyamines generally increase hydration, driving proteins into more compact configurations, and urea generally has the opposite effect. Gross alterations in the folding of a protein may also affect enzymatic activity, but that is an improbable explanation for changes such as those observed in the present studies. Thus, even if a methyamine favors compaction of an enzyme and urea has the opposite effect, the methyamine need not always oppose the effect of urea on enzyme activity (13). More subtle effects of altered hydration of the enzyme proteins may be involved. These could include allosteric changes and altered hydration at the catalytic site, depending on the structure and catalytic mechanism of the particular enzyme. For example, a high concentration of polyethylene glycol \((M_r \geq 20000)\) reduces the \(K_m\) of hexokinase for glucose. The interpretation, based on knowledge of the molecular structure and catalytic mechanism of hexokinase, is that osmotic dehydration alters conformational changes associated with catalysis (14). Such analysis has also become feasible for aldose reductase with progress in analyzing its molecular structure and mechanism of catalysis (15). In what follows we review these findings for a clue to the atypical responses of aldose reductase to urea and methyamines.

Kinetic analysis (15) has revealed that the mechanism of carbonyl reduction by aldose reductase is complex:

\[
E \rightleftharpoons E\text{-NADPH} \rightleftharpoons \text{E-NADPH-RCHO} \rightleftharpoons \text{E-NADPH-RCHO} \rightleftharpoons \text{E-NADP^+} \rightleftharpoons \text{E-NADP^+} \rightleftharpoons E
\]

where \(E\) is aldose reductase. \(E\) indicates kinetically significant conformational changes of the two binary E-nucleotide complexes and corresponds to the movement of a crystallographically identified nucleotide-clamping loop involved in nucleotide (NADP\(^+\)) exchange (16). In that study (15) the complete set of rate constants was determined for the substrate D-xylose, leading to the conclusion that the Michaelis constant, \(K_m\), includes contributions from numerous steps in the reaction scheme (15). Thus, \(K_m\) is not simply the binding affinity of the substrate for the \(E\)-NADPH complex, as it might be in a less complicated reaction, and changes in \(K_m\) do not necessarily reflect only changes in affinity of the enzyme for the substrate. The \(K_m\) for D-xylose is controlled to a large degree not only by the on-rate for aldehyde binding (\(E\)-NADPH \(\rightarrow\) \(E\)-NADPH-RCHO), but also by the rate constant for hydride transfer (\(E\)-NADPH-RCHO \(\rightarrow\) \(E\)-NADPH-RCHO \(\rightarrow\) \(E\)-NADP^+ \(\rightarrow\) \(E\)-NADP^+)). Accordingly, the explanation for the decrease in \(K_m\) caused by urea and the methyamines in the present study could involve alterations in one or more of these processes.

Mutation of a single amino acid (C298A) in recombinant human aldose reductase increases the \(K_m\) for D-xylose by 37-fold and also increases \(V_{max}\) (9). The increase in \(K_m\) is mainly caused by increase in the rate constant for \(E\)-NADPH \(\rightarrow\) \(E\)-NADP^+ resulting in relaxation of a tight binary E-nucleotide complex to a more weakly bound complex. We used the C298A mutant in the present studies to determine whether C298A might be important for the fall in \(K_m\) that unexpectedly was caused by urea and thus for the lack of counteraction. The results support that possibility. Urea increases the \(K_m\) of C298A for D-xylose (Figs. 1–3), which is similar to the effect of urea on many other enzymes, and is in striking contrast to the lowering of \(K_m\) of wild-type aldose reductase by urea (Figs. 1–5). With Dl-glyceraldehyde as substrate, urea neither raises nor lowers \(K_m\) (Figs. 4 and 5). The effects of the methyamines on \(K_m\) of C298A are similar to their effects on the wild-type enzyme (Figs. 1–5) and are consistent with the lowering of \(K_m\) by methyamines observed with many other enzymes (4). We conclude that C298A is involved in the decrease in \(K_m\) of wild-type aldose reductase that is caused by urea, but not the decrease in \(K_m\) caused by methyamines.

Counteraction involves additivity of the independent effects of urea and methyamines, which may result (when their effects are opposite in direction) in no net change in the \(K_m\). That may occur at a ratio of urea to methyamines close to 2:1 (3, 4). Betaine counteracts the effect of urea on the \(K_m\) of C298A for xylose in this classical fashion (Fig. 2), which is in striking contrast to the lack of counteraction with the wild-type enzyme (Fig. 2). Thus, with xylose as substrate, conversion of a single amino acid (C298A) alters the effect of urea so that it elevates, rather than decreases, \(K_m\) of aldose reductase, and sets the stage for counteraction by betaine.

On the other hand, with this mutant enzyme we do not observe classical counteraction with Dl-glyceraldehyde as substrate or with the methyamines other than betaine. In some cases the result with urea plus methyamine is intermediate between the effects of either alone, but the combination does not restore the control value. Thus, with Dl-glyceraldehyde as substrate (Fig. 5), betaine lowers the \(K_m\) of C298A, but urea alone does not change it, and the result with betaine plus urea is intermediate between those values. Similarly, with D-xylose as substrate TMAO has little effect on \(K_m\) (at least with the buffers used; see Results), whereas urea raises it, and the combined effect of urea plus TMAO is also intermediate between the effect of either alone. The result with GPC is somewhat different (Fig. 3). Although urea significantly elevates \(K_m\) for D-xylose and GPC lowers it, their effects are not additive, and \(K_m\) does not differ between urea alone and urea plus GPC. Thus, we observe counteraction between the effects of urea and some methyamines on \(K_m\) of the C298A mutant of aldose reductase, but not of wild-type aldose reductase.

C298 is part of the active site of aldose reductase, and during the catalytic cycle is involved in conformational changes that stabilize the \(E\)-NADP^+ complex. That allows wild-type aldose reductase to maintain a relatively low \(K_m\) (but also with a relatively low reaction rate). The C298A mutation greatly increases both \(K_m\) and \(V_{max}\) presumably by removing this constraint. Urea might further stabilize the \(E\)-NADP^+ complex in the wild-type enzyme, reducing both \(K_m\) and \(V_{max}\), but fail to have this effect on the mutant lacking C298. This explanation implies that urea stabilizes the \(E\)-NADP^+ complex in a relatively specific manner that is not counteracted by methyamines.

Perspective: The present phenomenological studies add to previous evidence that, although urea and methyamines may have opposite and counteracting actions on activities of some enzymes in vitro, the phenomenon is not general. That raises at least two questions:

(i) What are the atomic determinants of actions of urea and methyamines on particular enzyme activities? Although we have considerable insight into the mechanism by which urea and methyamines affect protein folding, that information does not readily translate into understanding of effects on enzyme activity.

(ii) What determines counteraction in living cells? We find the evidence that counteracting effects of urea and methyamines support the survival and function of organisms (4) and cells (5) to be convincing. However, if the effects of urea are counteracted by methyamines on some but not on other enzyme activities in living cells, questions arise as to which activities are affected in this fashion and which are not. Also, which effects are important for cell function and survival and why?