Human aldose reductase unfolds through an intermediate.

[version 1; peer review: 2 approved with reservations]

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Abstract

Background: Human aldose reductase (hAR) converts glucose to sorbitol under hyperglycemic conditions. Aldose reductase is first and rate limiting enzyme of polyol pathway. Under hyperglycemia, increased flux of glucose through this pathway has been implicated in development of secondary complication in diabetes. Due to this clinical implication, aldose reductase attracted considerable attention from drug discovery perspective. In spite of extensive characterization of the biochemical and structural context, little is known about the unfolding behavior of aldose reductase. This study reports equilibrium unfolding studies of human aldose reductase.

Methods: We carried out thermal and chemical induced equilibrium unfolding studies of human aldose reductase monitored by circular dichroism and tryptophan and ANS fluorescence spectroscopy.

Results: Thermal unfolding studies present a classical picture of two state unfolding from native to unfolded state. The data was used to derive thermodynamic parameters and study thermostability of aldose reductase. Urea and GuHCl induced equilibrium unfolding studies led us to discover an intermediate state, which gets populated at 3.5-4.0 M and 0.7-2 M of urea and GuHCl, respectively. Thermodynamic parameters from chemical induced unfolding are in agreement with those obtained from thermal unfolding.

Conclusion: This study revealed that aldose reductase unfolds from native to unfolded state via an intermediate. Assessment of thermodynamic stability of native, intermediate and unfolded state shows that three states are separated by significant energy barriers that ensure cooperativity of unfolding. As hAR functions in cells which are under osmotic and oxidative stress, these in vitro findings may have implications for its native conformation under physiological state.

Keywords

Aldose reductase, Protein unfolding, Folding intermediate, Cooperativity, Tryptophan fluorescence, ANS fluorescence, Thermal unfolding
Abbreviations
GuHCl, guanidine hydrochloride; TCEP, (tris(2-carboxyethyl)phosphine); ANS, 8-anilino-1-naphthalenesulfonic acid ammonium salt; IPTG, isopropyl β-D-thiogalactopyranoside; Trp, tryptophan.

Introduction
Human aldose reductase (hAR) (EC 1.1.1.21) is an NADPH-dependent oxidoreductase that belongs to super family of aldo-keto reductases. Being the first and rate limiting enzyme of polyol pathway, hAR converts glucose to sorbitol. Under hyperglycemic conditions, the polyol pathway is up-regulated and a significant proportion of glucose gets fluxed through this pathway, which leads to accumulation of sorbitol, consumption of NADPH and redox imbalance of NADPH/NADP⁺ ratio. All these factors have been linked with various tissue based pathologies associated with secondary complications of diabetes mellitus. Due to its clinical importance, hAR has been widely studied from the perspective of development of potent inhibitors so as to prevent or delay the onset of secondary diabetic complications.

Extensive information is available in the literature about the structure and function of hAR, particularly related to active site of hAR from ultra-high-resolution crystal structures with a number of potential inhibitors, flexibility in the hAR binding site pocket and the thermodynamics of closing/opening of the specificity pocket within binding site pocket of hAR. Nevertheless, there is little investigation related to the folding/unfolding mechanism of hAR. Understanding the capability of a polypeptide chain to spontaneously fold into a compact tertiary structure on biological relevant time scale is a long-standing challenge in protein science.

Under physiological conditions, protein structure fluctuates among different native conformations separated by close free energy barriers. Since hAR activity leads to sorbitol accumulation, leading to osmotic stress; it seems to function under stress conditions which might perturb its native conformation ensemble. Here we report on thermally and chemically induced unfolding studies of hAR. Thermal unfolding revealed simple two-state transition whereas chemical induced unfolding led us to discover an intermediate state during hAR unfolding.

Methods
Materials
All chemicals were reagent grade and purchased from Sigma-Aldrich.

Protein purification
The hAR cDNA cloned into expression vector pET-15b (Novagen) was a kind gift from Dr. Alberto Podjarny (Department of Integrated Structural Biology, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS, INSERM, France). The plasmid, coding for a hexahistidine-tagged protein, was expressed into E. coli strain BL21 (DE3) (Novagen). The cells with recombinant plasmid were grown with 100 μM ampicillin at 37°C to an OD₆₀₀ nm value of 0.7 and protein expression was induced by adding 1 mM IPTG. Cells were grown for further 3 hours at 37°C. All further operations were carried out at 4°C unless otherwise stated. Cells were centrifuged, re-suspended and lysed by sonication. A Ni-NTA affinity column (GE Healthcare) was used for protein purification. The material used for stationary phase for the column was Ni-Sepharose and the flow rate of column was adjusted to 0.5 ml min⁻¹. Imidazole and other salts were removed by repeated dialysis in 50 mM potassium phosphate, pH-7 buffer containing 50 mM NaCl. Protein concentration was estimated using the molar extinction coefficient and absorbance reading at 280 nm. The histidine tag from recombinant protein was removed by thrombin (4 units of thrombin per mg of recombinant protein at room temperature for 3 hours). Cleaved protein was passed through the Ni-NTA column, and purified protein without tag was collected as flow-through. Enzyme activity was checked as per standard assay.

Homogeneity and molecular weight of hAR with and without histidine tags was analyzed under denaturing conditions on 15% SDS-PAGE. Purified hAR was stored at -20°C for further studies.

Thermal unfolding
Thermal unfolding was carried out at a final concentration of 2.8 μM protein in 50 mM potassium phosphate buffer, pH 7.0 containing 50 mM NaCl and 0.1mM TCEP. Transition between 20-70 °C was followed using a far-UV circular dichroism (CD) signal at 222 nm by using 0.1 cm path length cuvette at sampling rate of 1.0 °C min⁻¹ in a Jasco J-810 spectropolarimeter. Buffer blank was duly subtracted before reporting the change in ellipticity (millidegree) at 222 nm.

Chemical induced unfolding
Samples with 1.4 μM protein concentration were prepared in phosphate buffer (described earlier) containing different concentrations of GuHCl/urea. Samples were incubated for 12 hours to reach equilibrium at 25°C after which no change in signal occurred either in fluorescence or CD spectra. Trp fluorescence (excitation at 295 nm and emission recorded between 300 nm to 400 nm) and ANS fluorescence (excitation at 370 nm and emission recorded between 400 nm to 600 nm) measurements were performed using a Hitachi F-7000 fluorescence spectrophotometer for GuHCl samples and Jasco J-815 spectropolarimeter for urea samples. Far-UV CD measurements were performed using Jasco J-810 spectropolarimeter for guHCl samples and Jasco J-815 spectropolarimeter for urea samples. Quartz cuvette of 1 cm and 0.5 cm path length were used for fluorescence and CD measurements respectively. All measurements were done at 25 °C. Spectra were reported as ellipticity (millidegree) after baseline correction.

Heat capacity change (ΔCₚ) calculations
ΔCₚ value for unfolding of hAR was calculated from change in accessible surface area (ΔASA) according to Equation 1.

\[
\Delta C_p = -251 + 0.19 \times ([\Delta ASA])
\]

(Equation 1)

ProtSA web server was used to calculate change in accessible surface area from native to unfolded conformation of hAR.
Data analysis
GraphPad Prism version 7.04 for Windows (GraphPad Software, La Jolla, California) was used for analysis of thermal and chemical induced unfolding data on the basis of two and three state model respectively as described in following sections.

Thermal unfolding
Data was fitted by least square analysis to Equation 2\(^\text{19}\).

\[
\begin{align*}
Y &= \frac{(A_y + b_y \times T) \times \exp \left( \frac{\Delta H_u}{R} \times \frac{1}{T_T} \right)}{1 + \exp \left( \frac{\Delta H_u}{R} \times \frac{1}{T_T} \right)} \\
\text{Equation 2}
\end{align*}
\]

Where \(A_y \) and \(A_u \) are native and unfolded baseline intercepts respectively and \(b_y \) and \(b_u \) are native and unfolded baseline slopes respectively. \(\Delta H_u \) is enthalpy change at melting temperature \((T_m)\). \(T \) is absolute temperature and \(R \) is the gas constant.

Calculation of \(\Delta G \) values for transition region
Signal for native \((Y_n = A_y + b_y \times T)\) and unfolded baseline \((Y_u = A_u + b_u \times T)\) for every point in transition region was calculated from Equation 2. If \(Y \) is signal for a particular point in transition region, then fraction of unfolded protein \((F_u)\) at this point is given by Equation 3.

\[
F_u = \frac{Y_n - Y}{Y_n - Y_u} \\
\text{Equation 3}
\]

The equilibrium constant \((k)\) can be calculated from relative population of species using Equation 4.

\[
k = \frac{F_u}{1 - F_u} \\
\text{Equation 4}
\]

\(\Delta G \) can be calculated as a function of temperature using Equation 5.

\[
\Delta G = -RT \ln k \\
\text{Equation 5}
\]

Thermal stability curve
The thermal stability curve of hAR was constructed on the basis of Equation 6–8\(^\text{14}\).

\[
\begin{align*}
\Delta H_T &= \Delta H_u + \Delta C_p (T - T_u) \\
\Delta S_T &= \frac{\Delta H_u}{T} + \Delta C_p \times \ln \left( \frac{T}{T_u} \right) \\
\Delta G_T &= \Delta H_u \times \left[1 - \frac{T}{T_T} \right] + \Delta C_p \times \left[ T - T_u - \ln \left( \frac{T}{T_T} \right) \right] \\
\text{Equation 6} \\
\text{Equation 7} \\
\text{Equation 8}
\end{align*}
\]

Where \(\Delta H_u \) and \(\Delta S_T \) are enthalpy and entropy change respectively at temperature \(T \) with reference to \(T_u, T_n, T_i, \) and \(T_T \) are the temperatures at which \(\Delta H, \Delta S, \) and \(\Delta G \) are zero respectively. \(\Delta G, \) is the stabilization free energy of the native state relative to the unfolded state.

Chemical induced unfolding
Data was fitted by least square analysis to Equation 9\(^\text{31}\).

\[
\begin{align*}
Y &= \frac{(A_y + b_y \times T) \times \exp \left( \frac{\Delta H_u}{R} \times \frac{1}{T_T} \right)}{1 + \exp \left( \frac{\Delta H_u}{R} \times \frac{1}{T_T} \right) + \frac{\Delta H_u}{R} \times \frac{1}{T_T} \times \exp \left( \frac{\Delta H_u}{R} \times \frac{1}{T_T} \right) \times \exp \left( \frac{\Delta H_u}{R} \times \frac{1}{T_T} \right)} \\
\text{Equation 9}
\end{align*}
\]

Where \(A_y, A_u, A_i, \) and \(A_f \) are the native, unfolded and intermediate baseline intercepts, respectively and \(b_y, b_i, \) and \(b_f \) are the native, unfolded and intermediate baseline slopes, respectively. \([D]\) is denaturant concentration in molar. \(m_{p(i)}, \) and \(m_{p(f)} \) are denaturant gradient for native to intermediate and intermediate to unfolded state respectively. \(\Delta G_{N(I)}, \) and \(\Delta G_{I(U)} \) are stabilization free energy of intermediate state relative to native and unfolded state respectively.

Results
Thermal unfolding monitored by far-UV CD
Change in ellipticity at 222 nm fitted well on the basis of a two-state model (Figure 1A). This analysis gave values for \(\Delta H, \) and \(T_T \) which along with \(\Delta C_p \) value calculated from Equation 1 were used for non-linear regression of transition region (±5 kJ mol\(^{-1}\)) to Equation 8 (Figure 1C). Values of \(\Delta H \) and \(\Delta S \) were calculated over extended range of temperature by using Equation 7 and Equation 8, respectively (Figure 1D). Thermal stability curve is extrapolation of transition region assuming constant \(\Delta C_p \) during unfolding transition (Figure 1E). The relationship between \(T_T, T_u, T_i, \) and \(\Delta G (T_f - T_i) = \Delta G / \Delta C_p \) is presented in Figure 1F. Thermodynamic parameters obtained from analysis of thermal unfolding data are listed in Table 1. All raw data are available as Underlying data\(^\text{35}\).

Chemical induced unfolding monitored by fluorescence
There are six Trp residues in hAR, out of which four are part of the hydrophobic active site pocket in the core of the \(\beta\)-barrel and two are buried in alpha helices surrounding the barrel. Their fluorescence provided global signal of change in tertiary structure. ANS has been extensively used as a probe for non-native, partially unfolded conformations of protein. The binding of ANS to hydrophobic regions results in a significant enhancement of ANS fluorescence and a pronounced blue-shift of the \(\lambda_{max} \) of the fluorescence emission profiles of hAR equilibrated with different concentrations of denaturants are presented in Figure 2 (Figure 2A and Figure 3A for urea and GuHCl, respectively). A plot of \(\lambda_{max} \) against denaturant concentration indicated cooperative transition from native to unfolded state (Figure 2B and Figure 3B for urea and GuHCl, respectively). In case of ANS fluorescence, significant blue-shift of around 20 nm and 10 nm from the native to intermediate state was observed for urea and GuHCl, respectively (Figure 2B and Figure 3B for urea and GuHCl, respectively).
Table 1. Thermodynamic parameters derived from equilibrium unfolding transitions of hAR during thermal and Chemical induced unfolding studies using different probes.

<table>
<thead>
<tr>
<th>Chemical unfolding</th>
<th>Probe</th>
<th>$\Delta G_u^m$ (KJ mol$^{-1}$)</th>
<th>$\Delta C_p^m$ (KJ mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta C_p$ (KJ mol$^{-1}$ M$^{-1}$)</th>
<th>$\Delta H_g$ (KJ mol$^{-1}$)</th>
<th>$\Delta S_m$ (KJ mol$^{-1}$)</th>
<th>$T_g$ (K)</th>
<th>$T_h$ (K)</th>
<th>$T_s$ (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GuHCl [F$_{314}$]</td>
<td>9.47 ± 1.69</td>
<td>58.34 ± 1.14</td>
<td>48.34 ± 7.69</td>
<td>23.56 ± 0.46</td>
<td>0.16</td>
<td>2.48</td>
<td></td>
<td></td>
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<tr>
<td>GuHCl-ANS [F$_{480}$]</td>
<td>16.48 ± 0.54</td>
<td>57.26 ± 3.33</td>
<td>38.99 ± 1.15</td>
<td>22.38 ± 1.29</td>
<td>0.42</td>
<td>2.56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea [F$_{314}$]</td>
<td>30.88 ± 4.47</td>
<td>35.04 ± 4.16</td>
<td>13.11 ± 2.01</td>
<td>7.99 ± 0.84</td>
<td>2.35</td>
<td>4.49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea-ANS [F$_{480}$]</td>
<td>28.61 ± 2.39</td>
<td>38.6 ± 2.14</td>
<td>12.1 ± 1.13</td>
<td>8.95 ± 0.46</td>
<td>2.36</td>
<td>4.32</td>
<td></td>
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<tr>
<td>GuHCl [\Theta_{219}]</td>
<td>13.68 ± 5.78</td>
<td>34.13 ± 3.75</td>
<td>32.17 ± 11.63</td>
<td>14.9 ± 1.58</td>
<td>0.43</td>
<td>2.29</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Urea [\Theta_{214.8}]</td>
<td>30.53 ± 6.16</td>
<td>24.43 ± 6.25</td>
<td>14.12 ± 3.03</td>
<td>5.99 ± 1.30</td>
<td>2.16</td>
<td>4.08</td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Thermal unfolding</th>
<th>Probe</th>
<th>$\Delta G_u$ (KJ mol$^{-1}$)</th>
<th>$T_g$ (K)</th>
<th>$\Delta H_g$ (KJ mol$^{-1}$)</th>
<th>$\Delta S_m$ (KJ mol$^{-1}$)</th>
<th>$T_h$ (K)</th>
<th>$T_s$ (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature [\Theta_{222}]</td>
<td>50.25</td>
<td>329 ± 0.01</td>
<td>17.57</td>
<td>779.20 ± 15.36</td>
<td>2.37</td>
<td>248</td>
<td>284.65</td>
</tr>
</tbody>
</table>

Figure 1. Thermal unfolding studies of human aldose reductase (hAR) monitored by far-UV circular dichroism. (A) Change in ellipticity at 222 nm plotted as a function of temperature. (B) Fraction of protein folded (green dots) and unfolded (red dots) plotted against temperature. (C) Portion of transition curve used in van’t Hoff analysis. (D) Plots of $\Delta T$ and $\Delta S$ as function of temperature. (E) Thermal stability curve of hAR. (F) Triangular relationship among $T_h$, $T_s$, and $\Delta G_s$. Explanation for $T_g$, $T_g'$, $T_h$, and $T_s$ is given in text. Dashed lines are extrapolations. Solid line represents fit to unfolding transition, filled symbols represent data points from unfolding experiments and red symbols represent outliers.

Plot of $I_{max}$ against denaturant concentration indicated presence of an intermediate during unfolding transition (Figure 2C and Figure 3C for urea and GuHCl, respectively). $I_{max}$ in case of ANS fluorescence fits satisfactorily on the basis of three-state model (Figure 2C2 and Figure 3C2 for urea and GuHCl, respectively). For both urea and GuHCl induced unfolding, Trp fluorescence emission intensity at 314 nm fits satisfactorily to three-state model. In case of ANS fluorescence, both $I_{max}$ and fluorescence emission intensity at 480 nm fit equally well on the basis of three-state model. Thus, Trp fluorescence emission intensity at 314 nm and ANS fluorescence emission intensity at 480 nm were analyzed on the basis of three-state model to evaluate thermodynamic stability of hAR (Figure 2D and Figure 3D for urea and GuHCl, respectively). The thermodynamic parameters obtained from fitting are listed in Table 1. Trp and ANS fluorescence clearly demonstrate presence of an intermediate state populated at 3.5-4.0 M and 0.7-2 M urea and GuHCl concentration respectively, apart from the native and unfolded states (Figure 2E and Figure 3E for urea and GuHCl, respectively).
Chemical induced unfolding monitored by far-UV CD

Unfolding profiles of hAR equilibrated at different denaturants concentrations in far-UV CD are presented in Figure 4A1 and 4A2 for GuHCl and urea, respectively. Thermodynamic stability of hAR was determined on the basis of three-state model by plotting change in ellipticity at 219/222 nm as a function of denaturant concentration (Figure 4B1 and 4B2 for GuHCl and urea, respectively). The transition determined by far-UV CD detected intermediate state at similar concentrations of denaturant as interrogated by fluorescence spectroscopy. All three states can be clearly distinguished from Far-UV CD profiles (Figure 4C1 and 4C2 for GuHCl and urea, respectively). Thermodynamic parameters derived from far-UV CD data are listed in Table 1.
Figure 3. GuHCl-induced unfolding studies of human aldose reductase (hAR) monitored by fluorescence. (A1) Trp fluorescence scans and (A2) ANS fluorescence scans. (B1) $\lambda_{\text{max}}$ (Trp fluorescence) and (B2) $\lambda_{\text{max}}$ (ANS fluorescence) against [GuHCl]. (C1) $I_{\text{max}}$ (Trp fluorescence) and (C2) $I_{\text{max}}$ (ANS fluorescence) against [GuHCl]. (D1) $I_{295/314}$ (Trp fluorescence) and (D2) $I_{370/480}$ (ANS fluorescence) against [GuHCl]. (E1) Trp fluorescence and (E2) ANS fluorescence of samples in native (green), intermediate (cyan) and unfolded state (red). Solid lines represent fit to the unfolding transitions, filled symbols represent data points from unfolding experiments, red symbols represent outliers.

Discussion

The intermediate state with enhanced ANS fluorescence and significant blue shift of $\lambda_{\text{max}}$ pointed to an intermediate state with some sort of ‘molten’ nature during hAR unfolding. Far-UV CD studies strongly suggest that the intermediate state retains significant secondary structure during urea- and GuHCl-induced unfolding.

During chemical induced unfolding hAR unfolds through an intermediate state which is absent during thermal unfolding. Moderate concentration of denaturant is known to stabilize native or intermediate state\textsuperscript{17}. Absence of such stabilizing agent may be the reason that the intermediate state was not detected during thermal unfolding.
In all three probes used in studying unfolding, value of \( \Delta G_{(N-I)} \) obtained is \(~30\) kJ mol\(^{-1}\) and \(~15\) kJ mol\(^{-1}\) for urea- and GuHCl-induced unfolding respectively while a \( \Delta G \) of \(~70\) kJ mol\(^{-1}\) is almost same for both denaturants (Table 1). Thus, while urea seems to stabilize the native state with respect to the intermediate state, GuHCl seems to stabilize the intermediate state with respect to the native state.

It is known that small molecules change the free energy landscape of protein upon binding by selectively stabilizing native or intermediate/unfolded state\(^{18}\). Difference in value of \( \Delta G \) obtained from thermal and chemical induced unfolding is \(~20\) kJ mol\(^{-1}\) (Table 1), which is most likely due to free energy of stabilization and destabilization by urea and GuHCl, respectively.

Values of \( \Delta G_{(N-I)} \) obtained from analysis of ANS fluorescence data are 16.48 and 28.61 kJ mol\(^{-1}\) for GuHCl and urea, respectively (Table 1), which indicate that intermediate state is not separated by a steep energy barrier from native state. Values for \( \Delta G_{(I-U)} \) obtained from ANS fluorescence are 57.26 and 38.6 kJ mol\(^{-1}\) for GuHCl and urea respectively (Table 1), which indicate that the intermediate state is separated from unfolded state by a high energy barrier. Thus, the intermediate state of hAR is close to its native state which makes it functionally more relevant.

In summary, equilibrium unfolding studies of hAR have led us to discover that hAR unfolds through an intermediate state, which is close to native state, and might have physiological relevance under hyperglycemic conditions in diabetes.

**Data availability**

**Underlying data**

Figs: data_f000_hAR_unfolding.zip. https://doi.org/10.6084/m9.figshare.8001998.v1\(^{15}\).

This project contains raw data for chemically and thermally induced unfolding studies on human aldose reductase.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

**Grant information**

The author(s) declared that no grants were involved in supporting this work.
Acknowledgements
We acknowledge Dr. Alberto Podjarny (Department of Integrated Structural Biology, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS, INSERM, France) for hAR plasmid as a kind gift, Dr. Yogendra Sharma, at CSIR-CCMB, Hyderabad and Chairperson, Department of Biotechnology, Panjab University, for helping in spectroscopic data collection. Gurprit acknowledges research fellowship from UGC, Govt. Of India (UGC science JRF).

References


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Vladimir Uversky

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In this manuscript, Gurprit Sekhon and Ranvir Singh describe the results of the equilibrium unfolding of human aldose reductase. The authors conclude that urea- and GuHCl-induced unfolding of this protein is characterized by the presence of partially folded intermediate. This is an interesting study with some potential. However, additional experiments are needed to provide more evidence for the existence of an intermediate state and also to provide better description of the structural properties of this protein.

1. The authors are encouraged to add near-UV CD spectroscopy to the arsenal of techniques utilized in their study. This will give very important information on the effect of denaturants on the tertiary structure of a protein. In fact, combined use of near- and far-UV spectroscopy is the accepted practice in studies on the conformational stability of proteins.

2. Quality of the reported far-UV CD spectra is very low. Spectra are very noisy and their utilization for the analysis of the effects of urea and GuHCl on secondary structure of human aldose reductase is questionable. The authors have to change settings to generate more reliable and less noisy spectra. Probably, protein concentrations should be increased too.

3. Far-UV CD spectra of completely unfolded forms induced by high concentrations of urea and GuHCl are very different, suggesting that these unfolded forms are not similar. Why?

4. Compactness of an intermediate state should be evaluated. This can be done by a whole host of hydrodynamic techniques.

5. The authors should make sure that the protein does not aggregate at the conditions promoting formation of a partially unfolded intermediate. This can be done by simple light scattering experiments.

6. Data shown in Figure 3 suggest that very low GuHCl concentrations cause very noticeable changes in some of the analyzed parameters. Similar behavior was described previously and was attributed to the aggregation of partially unfolded species. This phenomenon should be discussed.
7. The presence of partially folded intermediates can be visualized using “phase diagram” method described in previous work by Kuznetsova et al. The authors are encouraged to use this approach for the analysis of their data.

8. The authors are encouraged to reconsider the use of terms "denatured/denaturation" and "unfolded/unfolding". Typically, unfolding is attributed to the process resulting in the formation of coil-like conformation, whereas denaturation is referred to the elimination of functional tertiary structure. Obviously, these terms are not equivalent - molten globule is denatured, but is not unfolded. Furthermore, although temperature increase typically causes melting of a protein tertiary structure, temperature-denatured species are often rather compact and preserve high levels of secondary structure.

9. The manuscript contains some linguistic issues and errors and definitely needs editing.

References

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Protein physics; protein folding; partially folded proteins; folding intermediates; protein misfolding' protein aggregation; conformational diseases; intrinsically disordered proteins

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
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Jonathan W. Mueller
Institute of Metabolism and Systems Research (IMSR), University of Birmingham, Birmingham, UK

Drs Sekhon and Singh have presented an in-vitro study "Human aldose reductase unfolds through an intermediate" to F1000Research to be considered for indexing. Foremost, the authors should describe the newly described intermediate more with respect to its potential physiological relevance to stress the relevance of this study.

Please remove "ultra" from any description for protein crystal structures, but add the actual resolution of that structure, 0.66 Å from Ref. 5, and explain what additional features were derived from these, compared to average crystal structures.

"Understanding the capability of a polypeptide..." - this sentence only has limited linkage to the sentence beforehand and should be deleted. Instead, novel concepts of protein folding and stability should be briefly covered.1, 2

Figure 1:
- A) it is not clear what the blue and red dots mean.
- B) Please do not use green and red as colors.

Figure 2:
- A+B) Please label the different graphs within the diagrams.

The manuscript needs careful copy-editing.

References

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes
If applicable, is the statistical analysis and its interpretation appropriate?  
Yes

Are all the source data underlying the results available to ensure full reproducibility?  
Partly

Are the conclusions drawn adequately supported by the results?  
Yes

**Competing Interests:** No competing interests were disclosed.

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