Interactions of α-Chymotrypsinogen A with Some Alkylureas

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The interactions of α-chymotrypsinogen A with urea, methyl-, N,N'-dimethyl-, ethyl-, N,N'-diethyl-, and propylurea were studied by means of calorimetry and circular dichroism. It has been found that the enthalpies of interaction of the alkylureas, with the exception of methylurea, with α-chymotrypsinogen A are distinctly from those of urea. Thus the transfer of the protein from water to aqueous urea and methylurea solutions is accompanied by release of heat, i.e., the overall reaction is exothermic, whereas the transfer of the same protein to solutions of other alkylureas is characterized by consumption of heat, i.e., the overall reaction is endothermic. By examining the far UV CD spectra it can also be concluded that the alkylureas are clearly less efficient denaturants than urea. The difference in behavior reflects the presence of the hydrophobic moiety in the urea molecule.

INTRODUCTION

α-Chymotrypsinogen A is a pancreatic protein composed of 245 amino acid residues arranged in a single polypeptide chain. It is cross-linked by five disulfide bridges, one of which includes the N-terminal residue. It is one of the most thoroughly studied of all proteins. The purpose of this investigation was to determine the denaturing action on α-chymotrypsinogen A of various alkylureas, i.e., ureas having one or more hydrogen atoms replaced by alkyl groups. As is well known, urea is a strong denaturant, and therefore it is interesting to know how alkylsubstitution affects its denaturing activity. Calorimetry and circular dichroism were chosen for investigating the activity of alkylureas. The first gives the enthalpy of denaturation, the second allows us to ascertain conformational changes brought about by the alkylureas. The two data sets are complementary and provide insight into the nature of the interaction. Previous studies of human serum albumin have shown that the alkylureas are clearly less efficient denaturants than urea. Similar studies with several other proteins, e.g., some heme proteins, and also α-chymotrypsinogen, using various methods, e.g., spectrophotometry and optical rotation, have also been reported. On the basis of the results obtained with α-chymotrypsinogen the conclusion was reached that the denaturing action of alkylureas was a function of the substituted aliphatic group and predominantly hydrophobic in character and that the mechanism of denaturation by alkylureas differed appreciably from that by unsubstituted urea. By calori-
metric studies it should be feasible to check this claim. Moreover, in our studies we have systematically covered the whole range of solubilities of individual alkylureas and thus obtained enough data for a proper evaluation of their denaturing action.

EXPERIMENTAL

Six times crystallized bovine pancreas \( \alpha \)-chymotrypsinogen A, free of salt, was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. The various ureas used were supplied by Fluka, Buch, Switzerland. For calorimetric measurements they were washed with reagent grade acetone, for CD measurements they were recrystallized from hot reagent grade benzene. Solutions of \( \alpha \)-chymotrypsinogen were prepared in distilled water. Protein concentration was determined by using \( E_{1\text{cm}}^{1\%} = 20.0 \) at 280 nm. The initial pH of protein solutions was about 4.5, and after addition of alkylureas it increased. The increase depended on alkylurea concentration and except at highest concentrations it was below 1.0\(^5\).

Calorimetric experiments were performed in an LKB Batch Microcalorimeter 10700 at 25°C. Initial protein concentrations were around 1.5\%/w/v. The two compartments in the reaction cell were filled with \((2.00 + \Phi)\) ml protein solution and 4.00 ml of alkylurea solution, respectively. \( \Phi \) is the protein displacement volume, i.e., the product of protein mass and its partial specific volume, 0.734 ml/g. The two compartments in the reference cell were filled with 2.00 ml of water and 4.00 ml of the same alkylurea solution. In some cases the protein was dissolved in more concentrated urea solution, and the enthalpy of transfer to a less concentrated urea solution was measured. The results obtained in this way were identical with those

![Figure 1. Enthalpies of transfer of \( \alpha \)-chymotrypsinogen A in aqueous urea and alkylurea solutions.](image)

**Figure 1.** Enthalpies of transfer of \( \alpha \)-chymotrypsinogen A in aqueous urea and alkylurea solutions.
found in the usual experiments. For each transfer at least two experiments were performed. Since the two cells were thermally not balanced, their thermal response differing by about 3.5%, a separate blank experiment had to be performed for each transfer. In the experiment the compartments in both cells were filled with 2.00 ml of water and 4.00 ml of denaturant solution. The apparent heat effect measured was accounted for in the real experiment. The experimental errors involved are relatively large, reflecting especially the lack of thermal balance as well as large enthalpies of mixing.

CD spectra were recorded at 25°C on a Roussel-Jouan Dichrographe Mark III. In the experiments silica cells of 0.01, 0.05, and 0.1 cm pathlength were used. The mean residue ellipticity \([\Theta]_{	ext{mrw}}\) was calculated using the following relation

\[
[\Theta]_{	ext{mrw}} = \frac{M_0 \Theta}{100 c l}
\]

where \(M_0\) is the mean residue molecular weight, 105; \(\Theta\) is the ellipticity, \(c\) is the concentration in g/cm³ and \(l\) is the pathlength in dm.

RESULTS AND DISCUSSION

The values of the enthalpies of transfer of \(\alpha\)-chymotrypsinogen from water to aqueous solutions of alkylureas are presented in Table I. For comparison the values for urea solutions are also included. In Figure 1 the corresponding plots are given. From Table I and Figure 1 it may be inferred that the enthalp-
| Final concn. (mol/dm$^3$) | $\Delta H_{\text{int}}$ (kJ/mol) |
|-------------------------|-------------------------------|
|                         | I                     | II                     | III                     | IV                     | V                     | VI$^a$                     |
| 1                       | $70 \pm 5^c$            | $35 \pm 2$            | $65 \pm 3$             | $190 \pm 10$           | $29 \pm 2$            |
| 2                       | $145 \pm 10$            | $45 \pm 3$            | $80 \pm 5$             | $280 \pm 15$           |
| 3                       | $200 \pm 15$            | $125 \pm 10$          | $370 \pm 30$           | $470 \pm 20$           |
| 4                       | $260 \pm 15$            | $230 \pm 15$          | $280 \pm 50$           | $530 \pm 20$           |
| 6                       | $480 \pm 30$            | $280 \pm 20$          | $2610 \pm 80$          |
| 8                       | $700 \pm 50$            | $350 \pm 20$          | $2610 \pm 80$          |

$^a$ The enthalpy values refer to the transfer from aqueous solutions at 25°C.

$^c$ Mean values of two or more experiments; ± are assumed error limits.

The enthalpies of transfer of the protein to aqueous urea and methylurea solutions are negative whereas all other enthalpies of transfer are positive. It has been shown$^7$ that the enthalpy of transfer is composed mainly of two contributions. The first is due to the unfolding of protein molecules caused by the action of denaturant and for all known cases it is positive$^7$. It is usually called $^7$the

![Figure 3. Far ultraviolet CD spectra of α-Chymotrypsinogen A in aqueous methylurea solutions.](image-url)
The second contribution stems from solvation changes, and it depends on the nature of the denaturant. In the case of urea it is negative throughout, and from the data in Table I it may be inferred that changes of solvation make the major contribution to the enthalpy of transfer. The same applies to methylurea solutions but the enthalpy values are considerably smaller, i.e., less negative, than in urea solutions. Substitution of a hydrogen atom with a methyl group apparently produces major changes in the interaction between the protein and the denaturant. Therefore it is not surprising that the enthalpies of transfer to ethylurea solutions are already positive, and that they increase with increasing denaturant concentration. The same behavior is observed with N,N'-dimethylurea. The enthalpies for propyl- and N,N'-diethylurea are given only for 1.0 mol/dm$^3$ solutions owing to their low solubility in water and they are positive as well. Butylurea is also sparingly soluble in water but even in dilute solutions the protein precipitates.

On the basis of the results obtained, it may be concluded that the entrance of hydrophobic groups into urea molecules produces drastic changes in the enthalpies of transfer. The contribution to the enthalpy is positive but on the basis of the enthalpy values alone more cannot be said, since nothing is known about the conformational changes involved. Thus the analysis of CD spectra is necessary for a more detailed interpretation of the calorimetric data.

Figure 4. Far ultraviolet CD spectra of α-chymotrypsinogen A in aqueous ethylurea solutions.
The recorded CD spectra are given in Figures 2—6. Discussion of the spectra will be based on the comparison of the spectrum of the native protein with those of the protein in urea and alkylurea solutions. The \([\theta] \text{ values for urea solutions are least negative through the whole concentration range studied, Figure 2. Since it is known that } \alpha\text{-chymotrypsinogen is gradually denatured by urea and in 8 mol/dm}^3 \text{ solution it is largely unfolded}^8, \text{ with the constraints imposed by the five disulfide bonds, methyl-, ethyl- and } N,N'-\text{dimethylurea are clearly less efficient denaturants than urea. In methylurea solutions the values follow the same pattern as in urea solutions, Figure 3, but they are clearly more negative which may be interpreted as diminished unfolding at the same denaturant concentration}^1. \text{ However, in ethylurea solutions, Figure 4, the } [\theta] \text{ values first decrease with increasing concentration and are below those for the native protein. They reach a minimum at about 6 mol/dm}^3 \text{ whereupon they increase. This indicates that the fraction of the ordered structure first increases with increasing ethylurea concentration and then at concentrations above 6 mol/dm}^3 \text{ it starts decreasing}^1. \text{ On the basis of the CD spectra which, owing to solvent absorption, are available only to around 220 nm, not more than this qualitative statement can be made. Similar behavior is observed with } N,N'-\text{dimethylurea, Figure 5. Thus owing to the presence of

![Figure 5. Far ultraviolet CD spectra of } \alpha\text{-chymotrypsinogen A in aqueous } N,N'-\text{dimethylurea solutions.}
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Figure 6. Far ultraviolet CD spectra of α-chymotrypsinogen A in aqueous N,N'-diethylurea and propylurea solutions.

the hydrophobic moiety, the denaturing action of the three alkylureas is different from that of urea. Depending on the size of the moiety and the concentration, the fraction of the ordered structure is diminished, remains the same or is increased. This conclusion is in essential agreement with previous findings with α-chymotrypsinogen⁴ where the action of alkylureas has been likened to that of corresponding alcohols. It should be noted that similar behavior has been observed with human serum albumin in solutions of the same alkylureas¹. In 1 mol/dm³ propylurea and in 1 mol/dm³ N,N'-diethylurea, Figure 6, the changes of the [6λ] values are small so that no conclusions regarding their action are feasible.

Returning now to the calorimetric data, it is possible to make additional comments on the enthalpy of transfer values found. The fact that the enthalpies of transfer to methylurea solutions are considerably less negative than those to urea solutions, although the extent of unfolding in the former is not much less, indicates that the difference is due to the positive contribution of the hydrophobic moiety to the enthalpy. In solutions of ethylurea and N,N'-dimethylurea the observed conformational changes of α-chymotrypsinogen involve an increase and a decrease in ordered structure, respectively, and it is not possible even to estimate their contribution to the enthalpies of transfer. However, considering the fact that the enthalpies increase with increasing
denaturant concentration it may be surmised that the hydrophobic interaction is dominant.

The combined application of calorimetry and CD spectroscopy has given useful information regarding the interaction of α-chymotrypsinogen with alkylureas and their denaturing activity. The data are in complete agreement with those obtained in the previous study of human serum albumin¹ and give an impetus to studies of other proteins.

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POVZETEK

Interakcije α-kimotripsinogen A z nekaterimi alkilsečninami

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Interakcije α-kimotripsinogen A s sečnino, metil-, N,N'-dimetil-, etil-, N,N'-dietil- in propilsečnino so bile raziskane z uporabo kalorimetrije in cirkularnega dikroizma. Ugotovljeno je bilo, da so entalpije interakcije alkilsečnin z α-kimotripsinogenom A precej različne od entalpij interakcij za sečnino. Prehod proteина iz vode v raztopino sečnine in metilsečnine je eksotermen, medtem ko je pri ostalih alkilsečninah endotermen. Na osnovi spektrov CD pa lahko sklepamo, da so alkilsečnine manj učinkoviti denaturanti kot sečnina. Razlika v obnašanju odseva prisotnost hidrofobne skupine v molekuli sečnine.

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