# HYDROPHOBIC BONDS IN PEPSIN

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## Introduction

This article discusses the problem of hydrophobic interactions in pepsin, utilizing data from the literature and our own experimental results.

The concept of "hydrophobic bonds" (interactions) is more and more frequently called upon to explain the physicochemical properties of macromolecules. There are a few works, principally theoretical or qualitatively experimental, devoted to hydrophobic interactions in proteins. The theory of hydrophobic interactions is most extensively covered in the articles of Scheraga and Nemethy [1-3], who presented a statistical treatment of hydrophobic forces based on the models of structured water devised by Bernal [4-6] and Frank [7]. A hydrophobic interaction in a protein is understood to refer to the interaction of nonpolar residues of such amino acids as alanine, valine, leucine, cystine, methionine, phenylalanine, etc. According to Scheraga and Nemethy [2], the stability of such bonds or (in thermodynamic terms) the negative free energy of their formation results from a decrease in the ice-like character of water during formation of hydrophobic bonds. As is well known [8], dissolution of hydrocarbons (used to simulate nonpolar amino acids) is accompanied by a decrease in specific entropy of about 20 entropy units per mole and only a slight change in enthalpy (0-1 kcal/mole). The enthalpic constituent is responsible for the negative change in free energy during formation of hydrophobic bonds. The increase in entropy can be explained in the following manner: water is subject to continuous formation and melting of regions with an ordered structure similar to that in ice, in which the water molecules retain a roughly tetrahedral coordination produced by four directed intermolecular hydrogen bonds. However, severe stretching and breakage of the hydrogen bonds takes place at the same time, so that there are water molecules with from 0 to 4 hydrogen bonds. The relative number of water molecules with a given number of hydrogen bonds follows a Boltzmann distribution ($N \sim e^{-E/kT}$, where E is the energy of the molecule). Molecules with 3, 2, 1, or 0 hydrogen bonds can participate in interactions of the dipole-induced dipole type with nonpolar amino acid residues, their energy increasing in this case. According to the Boltzmann formula, the molecular distribution then shifts toward an increase in the number of molecules with saturated hydrogen bonds, i.e., the structuring (ice-likeness) of the water increases. It is understandable that the ice-likeness of the solution becomes greater with the contact surface between the water molecules and the nonpolar residues; conversely, the ordering of the solution decreases when the nonpolar residues aggregate, which leads to a negative change in free energy and is manifested in stability of such aggregated nonpolar segments of a protein. As Scheraga noted, aggregation may not be important for small hydrocarbon molecules, since the rotational degrees of freedom are lost in this case. However, such aggregation is more important in proteins, since the backbone of the polypeptide chain is to a large extent rigidly fixed.

Calculations have shown that the energies of hydrophobic and hydrogen bonds are comparable. The influence of hydrophobic bonds on the kinetic and equilibrium properties of proteins has been demonstrated and qualitatively evaluated in articles by Scheraga [9] and Scheraga and Nemethy [1-3]. In particular, these authors showed that hydrophobic bonds, in contrast to hydrogen bonds, do not weaken with rising temperature until a definite limit (65°) is reached and may even be somewhat strengthened. This also follows from the fact that solution of hydrocarbons (which we used as a model of hydrophobic bonds) is an exothermic process.

Experimental investigation of the influence of hydrophobic bonds on the kinetic and equilibrium properties of proteins is hampered by the complexity of segregating the effect of hydrophobic interactions in pure form. For example, the effect of the hydrophobic interactions in hemoglobin (whose existence was proved by determination of the structure of this compound [10]) are masked by the effect of the hydrogen bonds in the $\alpha$-helical segments of the molecule.

## Hydrophobic Interactions in Pepsin

Hydrophobic interactions apparently occur in purer form in pepsin than in other proteins. The present paper is an attempt to generalize the available data on the hydrophobic interactions in this compound.

Various investigations of pepsin have shown that this proteolytic enzyme has unusual physicochemical properties. It has a molecular weight of 34,500 and evidently consists of a single polypeptide chain containing 341-345 amino acid residues [11]. Of these, 60% are nonpolar (glycine, valine, leucine, isoleucine, alanine, methionine, etc.), 71 are dicarboxylic amino acids, 36 are free carboxylic residues, and only 4 are basic residues (one lysine, one histidine, and two arginines) [12]. The optical rotation and dispersion of optical rotation also show that pepsin does not have an $\alpha$-helical chain configuration [12-13]. The optical rotation of pepsin is small, while the $\lambda_c$-constant of the dispersion of optical rotation is 216 (a typical figure for denatured proteins) and is not altered by exposure to urea in concentrations of up to 4 M. Enzyme activity persists in this case [12-14], i.e., hydrogen bonds apparently do not participate in stabilizing the globular configuration. The pepsin molecule is folded into a compact globule by the hydrophobic interaction of the nonpolar amino acid residues [3, 14, 15]. This hypothesis has also been confirmed by studies of the kinetics of thermal denaturation of pepsin in ethanol solutions of different concentrations [16].

## Kinetics of Thermal Denaturation of Pepsin

Edelhoch investigated the kinetics of thermal denaturation of pepsin in alcohol solutions of different concentrations [16] and compared his results with those of a similar study of hemoglobin [17] (Table 1). As Edelhoch noted, the denaturation rate increases in both cases, but a rise in the alcohol concentration has opposite effects on the activation parameters of pepsin and hemoglobin. The decrease in $\Delta S^*$ (the change in activation entropy) for pepsin and the increase in this factor for hemoglobin indicate that the molecules of the former pass into a less-ordered state, while those of the latter pass into a more-ordered state. The influence of alcohol on $\Delta H^*$ and $\Delta S^*$ (where $\Delta H^*$ is the change in activation enthalpy) should depend on the relative contributions made by hydrogen and hydrophobic bonds. The differing character of the changes in $\Delta S^*$ and $\Delta H^*$ for pepsin and hemoglobin indicates that most of the amino acids in the hemoglobin molecule, which has a molecular weight of 34,000, are hydrogen-bonded in the native protein, while there are few hydrogen bonds in pepsin, which has a molecular weight of 34,500. It is obvious that saturation of the hydrogen bonds and weakening of the hydrophobic bonds occurs in hemoglobin when alcohol is added, while the pepsin molecule passes from a more-ordered into a less-ordered state as a result of breakage of the hydrophobic bonds.

Table 1. Influence of Ethanol on Kinetics of Thermal Denaturation of Pepsin and Hemoglobin

| pH | Temp. region, °C | Alcohol conc., % | ΔH* | ΔF* | ΔS* |
|---|---|---|---|---|---|
| | | | Pepsin | | |
| 5.90; 0.15 -NaCl | 41—49 | 0 | 95 | 226 | 23.0 |
| | 31—36 | 20 | 88 | 215 | 22.0 |
| | 21—29 | 40 | 52 | 104 | 21.2 |
| | | | Hemoglobin | | |
| 6.00—7.00 | — | 0 | 76 | 153 | 25.1 |
| | — | 20 | 107 | 264 | 23.5 |
| | — | 40 | 117 | 309 | 22.5 |

Table 2. Structural Parameters of Pepsin Molecule

| Protein modification | Radius of inertia, Å | Ratio of axes of equivalent ellipsoid of rotation | Axes of ellipsoid, Å | Vol., Å | Surface-to-volume ratio, $Å^{-1}$ |
|---|---|---|---|---|---|
| Native pepsin at pH 5.6 | 20.5 | 2 | 37<br>74 | 55000 | 0.26 |
| Pepsin + 40% ethanol (0.15-N NaCl) | 28 | 3 | 38<br>114 | 133000 | 0.15 |

Edelhoch's results indicating that both hydrogen and hydrophobic bonds contribute to the conformation of pepsin and hemoglobin are in good agreement with the data yielded by x-ray diffraction analysis of the structure of hemoglobin and by physicochemical studies of pepsin.

## Investigation of Structural Parameters of Pepsin Molecule

We employed diffuse scattering of x rays at small angles [18] to investigate the change in the structural parameters of the pepsin molecule during heating and when the solvent polarity was changed (Table 2). Ethanol was used as a nonpolar solvent. All the structural parameters of the pepsin molecule were markedly altered at an ethanol concentration of 40%. The sharp jump in the radius of inertia in a 40% alcohol solution was due to weakening of the hydrophobic interactions in the pepsin. The molecular volume greatly increased and the surface-to-volume ratio dropped from 0.26 to 0.15 $Å^{-1}$. In order to demonstrate that the conformational transition does not result from electrostatic interactions (it will be remembered that pepsin contains 71 dicarboxylic amino acids), we also made measurements with pepsin solutions in which these interactions were suppressed with 0.15-N NaCl and obtained the same results.

Since the hydrophobic regions of the pepsin molecule contain tryptophan, tyrosine, and phenylalanine (6, 8, and 14 residues, respectively), a change in the environment of the chromophoric groups of these amino acids should affect the absorption of pepsin solutions in the vicinity of 280 mμ. The change in absorption at 280 mμ paralleled that in the radius of rotation. The optical density jumped from 0.700 to 0.780 at an alcohol concentration of 40%.

Comparison of our results with those obtained by Edelhoch shows that the structural changes in pepsin parallel those in the heat and entropy of activation, while the sudden jumps

in the activation parameters of the molecule, i.e., its biological activity, are closely if not directly related to conformational transformations.

## Thermal Stability of Pepsin

As was mentioned in the introduction, hydrophobic bonds, in contrast to hydrogen bonds, are not weakened at elevated temperatures and may even be somewhat strengthened. In this connection, we expected to find the conformation of the pepsin molecule to be thermostable if it were actually produced by hydrophobic bonds. We demonstrated in a previous article [18] that the optical density of a solution remains unchanged when the temperature is raised from the ambient level to 80°.

Moreover, study of the scattering of x rays at small angles by a 2% pepsin solution at 70° shows that the geometric parameters of the heated molecule are identical to those at room temperature. This apparently is still the only example of thermostability of the hydrophobic bonds of a protein.

Pepsin is known to be comparatively thermostable [19]. It loses only 25% of its enzyme activity in solution when held at 56° for 6 h. Dry pepsin can be heated to 100–120° without loss of enzyme activity. The enzymatic stability of pepsin apparently results from the thermostability of the conformation of the polypeptide chain, which is held in a compact globule by the hydrophobic interaction of the nonpolar amino acids.

In connection with the thermostability of pepsin, it is interesting to note that the pepsin predecessor pepsinogen can be heated to the boiling point and will regain its potential activity when cooled in the absence of salt.

## Conclusions

1. We have considered the role of hydrophobic bonds in pepsin.

2. It has been demonstrated that various physicochemical studies of pepsin presuppose that its conformation is determined principally by hydrophobic bonds.

3. Pepsin has been used to confirm experimentally the hypothesis that the hydrophobic bonds in a protein are thermostable.

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