Reconstitution of Acid-denatured Catalase*

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The current interest in the reversibility of protein denaturation is prompted by the idea that, unlike the amino acid sequence, the secondary, tertiary, and quaternary structures of native protein molecules may not be genetically predetermined during biosynthesis. If this viewpoint is correct, unfolded polypeptide chains, on denaturation of proteins, should be able under favorable conditions to return to the original three-dimensional conformations with the same biological activity as the native proteins. The recent studies on ribonuclease by Anfinsen et al. (1) and White (2) have clearly shown that the denatured protein with the disulfide bonds broken can regain its physical, chemical, and enzymic properties through the reoxidation of the sulphydryl groups and the removal of the denaturing agent. Similar experiments on Taka-amylase and lysozyme have been reported by Isemura et al. (3-5). Very recently, Stellwagen and Schachman (6) and Deal, Van Holde, and Rutter (7, 8) succeeded in denaturing and reconstituting a more complex enzyme, aldolase, with low enzymic activity consisted of three peaks at 11.3, 7.6, and 4.15 S, which they attributed to the native molecule and half-size and quarter-size subunits. In this paper, we will report the denaturation and regeneration of bovine liver catalase, which has a molecular weight of 240,000.

This multichain enzyme can easily be cleaved into two subunits in acid medium. It then can be reconstituted after removal of the denaturing agent and recrystallized with full enzymic activity. Earlier, Samejima, Kamata, and Shibata (9) found that catalase began to dissociate at below pH 3.7 and the process was complete at pH 3.0. The molecular weight of the subunit as determined by sedimentation and diffusion analyses was 120,000, just half the size of the native molecule. Tanford and Lovrien (10), on the other hand, studied commercial preparations that gave a sedimentation component of 4.6 S or below at pH below 3.5 and above 11. Further, they found that the preparation with low enzymic activity consisted of three peaks at 11.3, 7.6, and 4.15 S, which they attributed to the native molecule and half-size and quarter-size subunits. In this paper, we will therefore reinvestigate the size and shape of both native and acid-denatured molecules by other hydrodynamic methods.

EXPERIMENTAL PROCEDURE

Material—Crystalline bovine liver catalase was prepared according to the method of Shirakawa (11). Since the article was published in Japanese, we will briefly describe the procedures here. Liver, 1 kg, freshly slaughtered, was minced twice with a meat chopper, then mixed with 1 liter of cold distilled water, and kept at 4° overnight with occasional stirring. To the extract (after centrifugation) was slowly added cold acetone (0.8 volume per volume extract) at −7 to −2°. The precipitate was resuspended in about 500 ml of cold water, which gave a clear dark red solution after filtration or centrifugation. The filtrate or supernatant was half-saturated with cold ammonium sulfate, and the precipitate was redissolved in a minimal amount of cold water, immediately followed by centrifugation to remove insoluble residues. Usually, crude crystals appeared in a day or so. If it was necessary, a small amount of saturated ammonium sulfate was added to seed the crystallization. The crude crystals were redissolved in water at pH 7.4 by adding a few drops of 0.2 N NaOH. Recrystallization was effected by adjusting the pH to 5.4 with saturated KH₂PO₄ solution. Needle-like, plate-like, or hexagonal crystals were obtained, which were stored as a suspension in water at 0° until used. All chemicals used were reagent grade.

Methods—The concentrations of the catalase solutions were determined spectrophotometrically, with the value A₂₄₀ = 13.5 at 405 mμ, which corresponds to a molar extinction coefficient of 3.24 × 10³ (based on a molecular weight of 240,000). The latter was obtained on the basis of dry weights of two preparations after heating at 105° for more than 20 hours, and the two determinations agreed within 1%. The absorbance of the reconstituted protein (see "Results") was determined independently and found to be 13.4, which was essentially the same as that of the native catalase. The activity of catalase was measured by the method of von Euler and Josephson (12); the rate of decomposition of H₂O₂ was expressed in terms of Kat. f. (Katalase-fähigkeit) at 0°:

\[ \text{Kat. f.} = \log_{10} \left( \frac{X_0}{X} \right) / 50 \, \text{W} \]

in which X₀ and X represent the concentration of H₂O₂ at times zero and t, W is the concentration of catalase (grams per ml) and t is the time of reaction in minutes. The Kat. f. of our preparations varied between 32,000 to 35,000, which corresponded to the reaction rate, k₁ of 1.5 to 1.6 × 10⁻⁴ sec⁻¹ at 0°.

Sedimentation Analyses—Sedimentation coefficients of the proteins were measured with a Spinco model E analytical ultracentrifuge at 59,780 r.p.m. and at 4° or 20°. Molecular weights were determined by sedimentation equilibrium experiments in a multichannel cell (13) at both 4° and 20°. Photographic plates were analyzed with a Nikon microcomparator. The partial specific volume of native and denatured catalase was assumed to be 0.730 (14).
Viscometry—Intrinsic viscosities were measured with an Ubbelohde-type viscometer with flow times of about 350 to 1300 seconds for water at 4°C. Density corrections were applied to all intrinsic viscosities according to the suggestion by Tanford (15).

Spectrophotometry—The spectra of the protein solutions were obtained with an Optica spectrophotometer. The kinetic study of the Soret band, however, was made with a Zeiss spectrophotometer.

Sulfhydryl Group Determinations—The number of sulfhydryl groups in both native and denatured proteins was determined by spectrophotometric titration at 255 m⊥ with CMB (16).

RESULTS

Size and Shape of Catalase Molecule

Sedimentation and Viscosity—In Fig. 1 are shown the sedimentation velocity patterns of native, denatured, and reconstituted catalase. Unlike the commercial lyophilized preparations, our preparation shows only a single peak and so does the acid-denatured protein. Between pH 3.7 and 3.0, however, two components were observed (not shown here), one corresponding to the native protein and the other having the same sedimentation coefficient as at pH 3.0. Further, the relative area of the denatured component with respect to the native molecule increased with time, the rate of increase depending on the pH used.

The molecular weight of the native protein as determined by sedimentation equilibrium at 5,227 r.p.m. at 20°C was 240,000 (Table I), which agreed well with previously reported values of 248,000 (14), 225,000 (17), and 244,000 (18). The acid-denatured protein had a molecular weight of 120,000 by the same method at 8,225 r.p.m.; this again was in accord with the results obtained from sedimentation and diffusion measurements (9). Thus, the native protein molecule is cleaved into two physically indistinguishable subunits in acid medium.

The sedimentation coefficient, \(s_{20,w}\), of catalase drops from 11.4 S (18) to 4.5 S and its intrinsic viscosity, \([\eta]\) (at 4°C), increases from 0.039 to 0.21 dl per g on acid denaturation. Our denatured catalase has essentially the same \(s_{20,w}\) as Tanford and Lovrien's "4.6 S or below" component. In the absence of direct measurements of molecular weight by these authors, we are of course unable to determine whether their component could actually be half-molecule rather than quarter-molecule as they have inferred. The sedimentation coefficient of the protein at pH 3.0, as measured within 40 minutes after the rotor reached the chosen speed or after the solution had been stored for more than 15 hours at 4°C, were found to be identical. Likewise, no time dependence could be detected for the viscosity measurements. Further evidence that the dissociation at pH 3.0 is instantaneous will be presented in the spectroscopic study below.

The low intrinsic viscosity of native catalase implies a compact conformation of the protein molecule. By the Scheraga and Mandelkern treatment (19)

\[
\beta = \frac{NS\langle \eta \rangle}{6Mf(1 - \bar{n})} \tag{1}
\]

the \(\beta\) value was found to be \(2.28 \times 10^{4}\) which gave an axial ratio, \(p\), of about 5 to 6 for the equivalent hydrodynamic ellipsoid. From the conventional treatment

\[
[\eta] = \frac{\tau}{100} \tag{2}
\]

\(1\) The abbreviation used is: CMB, D-chloromercuribenzoate.

Fig. 1. Sedimentation velocity patterns of catalase at 4°C. A, native protein, 0.54% in 0.1 M phosphate buffer (pH 7.0); B, acid-denatured, 0.31% in 0.1 M KH₂PO₄ plus HCl (pH 3.0); C, reconstituted, 0.34% in 0.1 M phosphate buffer (pH 7.0). Photographs were taken at 30, 28, and 26 minutes for A, B, and C, respectively, after reaching the speed of 59,780 r.p.m. at schlieren diaphragm angles of 65-75°.
TABLE I

<table>
<thead>
<tr>
<th>Physical and chemical properties of catalase</th>
<th>Molecular weight (sedimentation-equilibrium)</th>
<th>( \beta ) at ( s_m )</th>
<th>[( \beta ) ( \times 10^4 )]</th>
<th>Sulfhydryl groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>240,000 ± 3,000</td>
<td>11.40 ± 0.15</td>
<td>0.039</td>
<td>2.26</td>
</tr>
<tr>
<td>Acid-denatured</td>
<td>120,000 ± 5,000</td>
<td>4.50 ± 0.02</td>
<td>0.21</td>
<td>2.52</td>
</tr>
<tr>
<td>Reconstituted</td>
<td>12.0 ± 0.15</td>
<td>0.046</td>
<td>2.26</td>
<td>6</td>
</tr>
<tr>
<td>Reconstituted and crystallized</td>
<td>250,000 ± 5,000</td>
<td>11.58 ± 0.03</td>
<td>0.042</td>
<td>2.26</td>
</tr>
</tbody>
</table>

The shape of the denatured catalase is more difficult to characterize, since the increase in intrinsic viscosity and decrease in sedimentation coefficient might arise from either the increase in asymmetry or the volume expansion of the molecule or both. The \( \beta = 2.52 \times 10^4 \) (Table I) value would indicate an axial ratio of more than 10 if the subunits were rigid. On the other hand, a randomly coiled polymer would characteristically have the same \( \beta \) value, following the Flory treatment. From other evidence presented below, however, we are led to believe that the subunits are grossly disorganized.

Absorption Spectra—in Fig. 2 are shown the absorption spectra of both native and acid-denatured catalase. In the native state the heme protein has the characteristic Soret band (at 405 \( \mu \)m) and other absorption maxima at 500, 535, and 622 \( \mu \)m. At pH 3.0, however, the Soret band is greatly diminished and the other three peaks have completely disappeared; instead, a new hump at 545 \( \mu \)m is observed. (Our results seem to differ from those reported by Osbahr and Eichhorn.) Their absorption curves for catalase were very much alike at neutral, acid, and alkaline pH, especially in their retention of the 620-\( \mu \)m peak.) On the other hand, the absorption maximum at 276 \( \mu \)m (due to aromatic groups) remained virtually intact on acid denaturation. These results clearly indicate a gross conformational change of the protein in acid medium. Further, the change seems virtually instantaneous. As soon as the solution was adjusted to pH 3.0, the first reading taken within 15 seconds for the Soret band was identical with that taken after the solution had been standing for 24 hours.

Sulphhydryl Groups—Catalase \( (9.6 \times 10^{-6} \text{ M}) \) was found to react with \( 6.32 \times 10^{-3} \text{ M CMB} \), indicating that 6 sulphhydryl groups were titratable in the native protein. At pH 3.0, however, \( 2.06 \times 10^{-3} \text{ M CMB} \) reacted with \( 1.30 \times 10^{-8} \text{ M protein} \), whereas \( 6.20 \times 10^{-3} \text{ M CMB} \) combined with \( 1.02 \times 10^{-8} \text{ M reconstituted protein} \). Thus, an additional 10 sulphhydryl groups were titratable for the acid-denatured catalase, and they were masked again on reconstitution.

Optical Rotatory Dispersion—in the succeeding paper (24), we will discuss in detail the drastic change in rotatory properties of catalase on denaturation. Suffice it to say that the characteristic Cotton effect due to the Soret band completely disappeared at pH 3.0. The helical content of the native protein was estimated to be about 50% by several rotation analyses, but at pH 3.0, the denatured protein still retained about 20% helix.

Enzymic Activity—At pH 3.0, catalase completely loses its enzymic activity.

Reconstitution of Catalase Subunits

Attempts to reconstitute the subunits of denatured catalase (at pH 3.0) by removing the hydrogen ions through dialysis...
led to the precipitation of the protein. Since the unfolded polypeptide chains in moderately concentrated solution easily entangle with one another, we have therefore kept the initial concentration well below 1% and resorted to the rapid dilution technique for the reconstitution process. This was done by pouring the catalase solution at pH 3.0 into 10 to 100 times volumes of 0.1 M phosphate buffer at pH 7.0 with vigorous stirring, the temperature being kept at 4°. By so doing the enzymic activity can indeed be regained partially. The extent of recovery was found to depend on many factors among which are the denaturation time, the dilution volume, and the renaturation time, as well as the concentration of the protein.

Denaturation Time—Although the physical and chemical properties of the acid-denatured catalase have been shown to be time-independent, the % recovery of enzymic activity does diminish on prolonged exposure to pH 3.0. It can be seen from Fig. 3 that after an initial rapid drop, the enzymic activity reaches a constant level of about 50% activity between 10 and 30 minutes. Beyond this limit, the recovery is further lowered until after about 3 hours, very little can be recovered, even though the size and shape appear to remain intact. Clearly, the active sites on the protein molecule must have been damaged by some side reactions in the acid medium, which, however, does not affect the gross conformation too much. The activity curve for the reconstituted and recrystallized catalase (to be discussed later) has the same characteristic shape as the native protein, a further indication that the two molecules are identical.

Renaturation Time—There is a rapid reconstitution of the subunits as soon as the acid-denatured catalase is poured into neutral phosphate buffer (pH 7.0) (Fig. 4). In 0.1 M buffer, the % recovery of enzymic activity essentially levels off within 30 minutes. On the other hand, at higher ionic strength, the recovery of activity is more gradual, but even in 0.5 M buffer, it approaches the same asymptote of about 50%. In all routine experiments, the renaturation time was therefore kept at 3 hours to insure maximal recovery.

Dilution Volume and Protein Concentration—The % recovery of enzymic activity is highly dependent on the initial concentration of catalase used (Fig. 5). In a concentrated solution, the unfolded polypeptide chains would easily entangle with one another. This perhaps explains the reduced recovery at high concentrations. On the other hand, it has been known that many native enzymes will also become partially inactivated when its concentration is extremely low. The optimal concentration in the present case appeared to be about 0.3%. The effect of the dilution volume was found to be less critical than that of the concentration. For an initial protein concentration between 0.3 and 0.6%, there appeared to be no difference, whether it was diluted 10 or 100 times.

Recrystallization of Reconstituted Protein—The very dilute solutions of renatured catalase can be concentrated by use of a DEAE-cellulose column previously equilibrated with 0.02 M phosphate buffer (pH 7.0). The protein layer can then be eluted with 0.1 M phosphate buffer (pH 7.0). About 50% by weight of the total protein could be recovered, whereas the rest was found to be adsorbed strongly to the column and it could not be washed off even with 0.5 M buffer. The use of 1 M buffer resulted in an additional partial recovery of the latter fraction of the protein, which, however, became turbid on standing. To characterize further this inactive portion of catalase, a 0.4% acid-denatured protein solution was reconstituted by 10 times

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**Fig. 3.** Effect of denaturation time of catalase at pH 3.0 on the % recovery of enzymic activity. Initial concentrations: ○—○, 0.35%; △—△, 0.31%; ■—■, 0.20%. ■ represents the reconstituted and recrystallized catalase undergoing a second cycle of denaturation. All measurements were made at 4° after rapid dilution with 100-fold (by volume) 0.1 M phosphate buffer (pH 7.0), followed by 3 hours of stirring.

**Fig. 4.** Effect of renaturation time of catalase on the % recovery of enzymic activity. Initial concentrations were 0.30 to 0.31%. The native protein was exposed to pH 3.0 for 15 minutes, followed by rapid dilution with 100-fold (by volume) phosphate buffer at pH 7.0; Curve A, 0.1 M and Curve B, 0.5 M buffer. Aliquots of the diluted solutions were assayed at intervals for enzymic activity.

**Fig. 5.** Effect of dilution volume of catalase on the % recovery of enzymic activity. Initial concentrations: Curve A, 1.0%; Curve B, 0.6%; Curve C, 0.3%; Curve D, 0.15%; Curve E, 0.075%. The native protein was exposed to pH 3.0 for 15 minutes and reconstituted by the rapid dilution method with various volumes of 0.1 M phosphate buffer (pH 7.0), followed by 3 hours of stirring.
rapid dilution. The resultant solution (0.04%) and also a native protein solution (0.04%) as a control were sedimented in the Spinco ultracentrifuge with 30-mm cells. The peak area of the reconstituted protein was only about one-half of that for the native protein. Further, precipitates were found at the bottom of the cell in the case of reconstituted protein and the concentration of the supernatant was about 0.02% which, however, had 100% recovery of the original enzymic activity. These findings seem to indicate that the inactive catalase are the aggregates of the denatured protein which are strongly adsorbed on the DEAE-cellulose column or easily precipitated on ultracentrifugation.

The reconstituted active catalase can be recrystallized by the same procedure as that described in the experimental methods. In Fig. 6 are shown the crystalline forms of both native and reconstituted catalase after three or four times recrystallization. The results are self-explanatory. From physical and chemical studies, the reconstituted protein has the same molecular weight, sedimentation coefficient, intrinsic viscosity, absorption spectrum, and number of titratable sulfhydryl groups as the native protein (Table I). Further, it has the same enzymic activity (95 to 100%) as the native protein on the basis of its Kat. f. determination. Thus, we are led to believe that the reconstituted catalase has regained its original conformation and biological activity through renaturation.

**DISCUSSION**

In this paper, we have shown that acid-denatured catalase can be reconstituted under suitable conditions in spite of its high molecular weight and multichain structure. The % recovery of enzymic activity on renaturation is, however, rapidly diminished after prolonged standing at pH 3.0, even though the size and shape of the subunits and the reconstituted protein molecule remain unchanged under similar conditions. Perhaps the simplest explanation of our results is similar to that of Linderstrom-Lang on the denaturation of β-lactoglobulin (23, 26), that is, the denaturation proceeds in two steps, one reversible and the other irreversible.

\[ N \xrightarrow{D} D' \]

The first process is determined by the equilibrium ratio between the two forms under prevailing conditions. For catalase, this step is extremely rapid at pH 3.0. On the other hand, the second process is slow in the present case, and it is perhaps directly involved with the active sites, the nature of which is still unknown. The rate of the second step for catalase is such that it becomes significant after about 30-minute exposure to pH 3.0.

Since native catalase has four heme groups, one is tempted to think that the protein molecule might consist of four polypeptide chains similar to those in hemoglobin. Thus, under different conditions, catalase might be cleaved into more than two subunits. Indeed, Samejima (18) has found three subunits in alkaline solution (pH above 12) on the basis of sedimentation and diffusion studies. Likewise, Samejima and Shibata (27) have reported that catalase can be dissociated into one-third and one-sixth size subunits in formamide solution and one-third size subunits in urea solution; further, in both cases the one-third size subunits can form dimers. At present, it is difficult to understand how the four heme groups distribute themselves among the three or six physically indistinguishable subunits. Work is being carried on to characterize further these subunits at high pH and also in urea solution by various physical and chemical methods, which, we hope, will eventually clarify this problem.

The fact that acid-denatured catalase still retains 20 to 25% helical content (from optical rotatory dispersion study (24)) seems to suggest that some strong local interactions in an incompletely disordered polypeptide chain might be responsible for the reconstitution process, which could have been initiated at those ordered helical regions even though most of the secondary, tertiary, and quaternary structures of the protein molecule are destroyed. For aldolase, Stellwagen and Schachman (6) found that \( \lambda _v \) of the Drude equation was 239 ms at pH 2.0 and 230 ms in 4 M urea, which also seemed to indicate the presence of some helices in the denatured protein, although without detailed analyses of their rotatory dispersion data it is difficult to make any reliable estimate of the helicity. On the other hand, Isemura et al. (3-5) did show that the denatured Takaamylase with or without the breaking of the disulfide bonds...
could be renatured with ease. Further, from their optical rotation data, the unfolded polypeptide chain appeared to be completely disordered. This strongly favors the current view that the refolding process of denatured proteins is predominantly dictated by the primary structure of the polypeptide chains. It seems too early to speculate further on this point until more studies of the protein renaturation process are undertaken.

**SUMMARY**

1. Bovine liver catalase (molecular weight, 240,000) is readily dissociated into physically indistinguishable subunits (molecular weight, 120,000) at pH 3.0 in 0.1 M KH₂PO₄ (plus HCl). This dissociation is accompanied by marked changes in hydrodynamic properties; the intrinsic viscosity increases from 0.039 to 0.21 dl/g at 4°C and the sedimentation coefficient, s₂₀,ᵦ, decreases from 11.4 to 4.5 S. Acid denaturation also results in a complete loss of enzymic activity and the disappearance of the absorption bands characteristic of heme proteins, in particular, a shift of the Soret band from a maximum at 405 mp to a small, broad peak at about 375 mp. Sixteen titratable sulfhydryl groups were found in the subunits as compared with only six in the native molecule. All these indicate a gross change in conformation on acid dissociation. This conclusion is further supported by optical rotatory dispersion studies (see the succeeding paper).

2. The dissociated catalase subunits can be reconstituted by rapid dilution of the pH 3.0 solution with a pH 7.0 phosphate buffer. The % recovery of enzymic activity, however, depends on many factors among which are: (a) the denaturation time; (b) the concentration of the protein; (c) the dilution volume; and (d) the renaturation time. Under optimal conditions, about 50% of the original activity can be regained. The renaturated protein solution can be concentrated by passing through a diethylaminoethyl cellulose column. About half of the protein can be eluted off the column. This recovered protein, however, has 95 to 100% enzymic activity. The reconstituted catalase was further characterized by its sedimentation-equilibrium molecular weight, sedimentation coefficient, intrinsic viscosity, absorption spectrum, titratable sulfhydryl groups, and optical rotatory dispersion. In all respects, it has the same physical and chemical properties as the native protein. Furthermore, the crystalline forms of the native and reconstituted catalase are also identical.

**REFERENCES**

Reconstitution of Acid-denatured Catalase
Tatsuya Samejima and Jen Tsi Yang