Biochimica et Biophysica Acta, 481 (1977) 420--430

BBA 68099

ON THE SPECIFIC ASSOCIATION OF PORCINE ERYTHROCYTE CATALASE CAUSED BY FORMATION OF DISULFIDE CROSS-LINKS

ATSUSHI TAKEDA and TATSUYA SAMEJIMA

Department of Chemistry, College of Science and Engineering, Aoyama Gakuin University, Setagaya-ku, Tokyo 157 (Japan)

(Received October 19th, 1976)

Summary

Porcine erythrocyte catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) has been purified from porcine blood by DEAE-cellulose column chromatography, ammonium sulfate fractionation and CM-cellulose column chromatography. The purified enzyme was found to associate into larger molecules than the native one when it was stored at 4° C for more than one week. The associated molecules can be detected by gel filtration on a Bio-gel A-1.5 m column and disc gel electrophoresis as well as ultracentrifugal analysis. Molecular weights of the associated catalase molecules were about 500 000, 750 000, 1 000 000 and so forth estimated by gel filtration and disc gel electrophoresis, corresponding to dimer, trimer and tetramer respectively, of a native molecule (monomer) with a molecular weight of about 250 000. The association of catalase molecules is found to be time-dependent and to proceed seemingly from monomer through dimer as an intermediate. From the effects of several thiol reagents or reducing reagents on the association process and spectrophotometric titration of SH groups, it is inferred that this specific association of porcine erythrocyte catalase is caused by formation of intermolecular disulfide cross-links due to air oxidation of SH groups in the protein moiety.

Introduction

It was first reported by Nagahisa [1] that porcine erythrocyte catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) can be crystallized from the purified enzyme solution in 0.1 M acetate buffer (pH 4.0) with a small amount of ammonium sulfate after several purification steps including ethanol/chloroform, acetone and ammonium sulfate fractionations. Although the erythrocyte catalase has been thus purified, little information is yet available on the characterization of this enzyme. Therefore, it seemed

worthwhile to investigate this enzyme further and in more detail, from the viewpoint of the elucidation of the relation between structure and function as well as comparative biochemistry of erythrocyte catalases from different sources. The purification of the enzyme from porcine blood was performed with some modifications according to the methods reported by Mörikofer-Zwez et al. [2] and Aebi et al. [3] for human and horse erythrocyte catalase. During the course of the present study we noticed that the purified enzyme showed first only a single band on disc gel electrophoresis, but multiple bands were invariably detected after storage of the preparation at 4° C for more than one week. We can deduce that intermolecular association is responsible for this phenomenon, yielding dimer, trimer and tetramer with respect to the native molecule, by forming disulfide cross-linkages from SH groups of cysteinyl residues in the protein moiety due to air oxidation. The present paper is concerned with brief description of purification procedure, process of specific intermolecular association, molecular weight determination of the associated molecules and effect of reducing and thiol reagents on the association.

Materials and Methods

Materials

DEAE-cellulose (DE-52), CM-cellulose and Bio-gel A-1.5 m were purchased from Whatman Ltd., Serva Co. and Bio-Rad Laboratories, respectively. Crystalline bovine liver catalase (EC 1.11.1.6) was prepared according to the method of Shirakawa [4]. Glutamine synthetase (EC 6.3.1.2) from *Saccharomyces cerevisiae* and adenosinetriphosphatase (EC 3.6.1.3) from *Bacillus stearothermophilus* were prepared according to the method of Hachimori et al. [5,6]. Aldolase (EC 4.1.2.13) (rabbit muscle) was purchased from Boehringer, Mannheim. All chemicals used were of reagent grade.

Enzyme assay

The catalase activity in the hemolysate and various enzyme preparations was measured according to the permanganate titration method developed by Bonnichsen [7].

Determination of protein

Protein concentrations were determined by the method by Lowry et al. [8] using crystalline bovine serum albumin as a standard.

Electrophoretic analysis

Polyacrylamide gel disc electrophoresis of catalase was carried out according to the procedure of Ornstein [9] and Davis [10]. The estimation of the molecular weight of catalase by disc gel electrophoresis was performed by the method of Hedrick and Smith [11]. SDS polyacrylamide gel electrophoresis of the proteins with 0.1% SDS was performed in the presence or absence of 0.1% 2-mercaptoethanol according to the method of Weber and Osborn [12], using 7.5% gels. The sample proteins were kept overnight in 10 mM phosphate buffer (pH 7.2) containing 1% SDS and 8 M urea in the presence or absence of 1% 2-mercaptoethanol prior to electrophoresis. The electrophoresis was carried out

at room temperature for 5 h at 5 mA per tube. The protein in the gel was stained with 1.0% Amido Black in 7% acetic acid. Densitometer tracings of gels were obtained at 570 nm using a densitometer, Model FD-AIV of Fujiriken Co.

Ultracentrifugal analysis

The measurements of sedimentation velocity were performed with a Hitachi model UCA-I analytical ultracentrifuge at 60 000 rev./min at 20°C. The protein concentrations in 50 mM phosphate buffer (pH 7.0) were 0.3—0.5%. The partial specific volume was assumed to be 0.73 [13].

SH group determinations

The number of SH groups in catalase molecule was determined by spectrophotometric titration at 250 nm with *p*-chloromercuribenzoate [14].

Preparation of porcine erythrocyte catalase

Porcine erythrocyte catalase was prepared according to the method of Mörikofer-Zwez et al. [2] and Aebi et al. [3] used for the human and horse enzyme with some modifications. The purification procedure will be briefly described here.

Hemolysis

Fresh porcine blood was centrifuged to remove the plasma and the erythrocytes were washed three times with cold physiological saline by centrifugations. The packed cells were hemolyzed by adding an equal volume of distilled water and subsequently stirring for 1 h at 4°C. The hemolysate was centrifuged to remove the cell stroma and some proteins, yielding a clear supernatant which was dialyzed overnight against distilled water at 4°C.

Chromatography on DEAE-cellulose

The dialyzed hemolysate was mixed with DEAE-cellulose (DE-52) which had been equilibrated with 3 mM sodium/potassium phosphate buffer (pH 6.8) followed by gentle stirring for 1 h at 4°C. Porcine erythrocyte catalase was adsorbed on the resin with this batchwise procedure. After the hemolysate/cellulose suspension had been washed with a large amount of the same cold buffer through a glass filter, the resuspended cellulose with the buffer was packed into a column (6 × 50 cm) and the remaining hemoglobin was washed out with the same buffer. The catalase was then eluted with 50 mM sodium phosphate buffer (pH 6.8) and active fractions were collected and pooled.

Ammonium sulfate fractionation

The pooled active solution was 35% saturated with ammonium sulfate by adding the solid salt with stirring at room temperature. After removing the precipitate by centrifugation, the supernatant was 50% saturated by adding solid ammonium sulfate. The precipitate was collected by centrifugation and dissolved with cold distilled water, followed by dialysis against 10 mM sodium acetate buffer (pH 4.8) overnight at 4°C.

CM-cellulose column chromatography

The dialyzed enzyme was adsorbed to a CM-cellulose column (2.5 × 30 cm)

TABLE I

PURIFICATION SCHEME FOR PORCINE ERYTHROCYTE CATALASE

Step	Volume (ml)	Activity *	Total activity	A_{405}/A_{280}	Total activity/A_{280}	Yield (%)
Hemolysate	6300	11.48	72 300	—	35.76	100
DEAE-cellulose	895	74.35	66 500	2.50	268.04	92
$(NH_4)_2SO_4$ fractionation (35—50%)	20	2021.74	40 400	1.09	610.27	56
CM-cellulose	85	344.35	29 300	1.18	820.73	41

* Activity is expressed as first order reaction constant per 1 ml of enzyme solution per min.

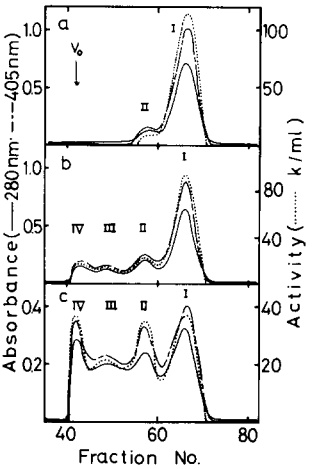
previously equilibrated with 10 mM sodium acetate buffer (pH 4.8). The column was washed with 100 mM sodium acetate buffer (pH 4.8) until the protein-free fraction was detected and then the catalase was eluted with 50 mM sodium phosphate buffer (pH 6.5). The active fractions were pooled and concentrated by ultrafiltration. The result of the above purification procedure is summarized in Table I. The concentrated catalase solution showed a Kat.f (Katalase-fähigkeit) value of 63 000 ± 3000 and an R.Z. (A_{405}/A_{280}) value of 1.18—1.23. However, this purified preparation showed a faint band besides a main band of the native catalase on disc gel electrophoresis.

Results

Detection of associated catalase molecules

At the final stage of the above purification an attempt was made to remove the minute quantity of a contaminating component from the native one using a Bio-gel A-1.5 m column. The above concentrated enzyme solution was immediately applied to a column of Bio-gel (2.5 × 90 cm), equilibrated and developed with 50 mM sodium phosphate buffer (pH 7.0). The elution profile of the enzyme from the Bio-gel column is shown in Fig. 1a (2—3 days were needed for complete elution). Besides a main peak which is designated as component I, another small component (designated as component II) with a larger molecular size than that of component I was detected. Component I could be completely isolated from component II in fractions after the peak position of component I. The pooled solution showed a homogeneity both in ultracentrifugal and disc electrophoretic patterns. This isolated component I was found to be native catalase with a molecular weight of one quarter million which will be described later.

Fig. 1b and 1c illustrate elution patterns of the enzyme from the Bio-gel column after 3 and 6 weeks' storage of the concentrated enzyme solution (3—5%) at 4°C, respectively. Two other new components with larger size (designated as components III and IV) were observed and their properties were found to be time-dependent. After 3 weeks component II increased and component III and IV newly appeared, whereas component I decreased (Fig. 1b). After 6 weeks it is evident that each relative amount of component II, III and



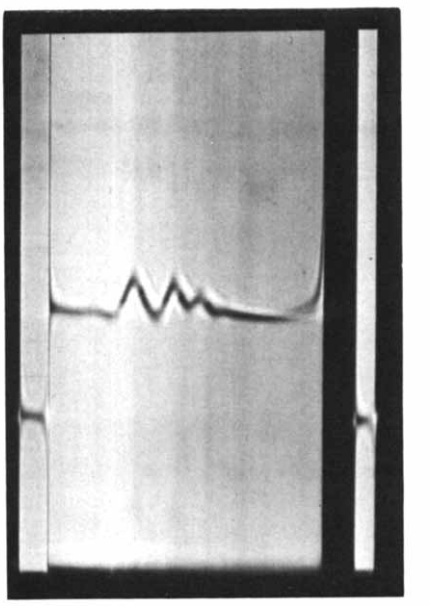


Fig. 1. Profiles of separation of the associated molecules by Bio-gel A-1.5 m column chromatography. Bio-gel A-1.5 m (column size 2.5 X 90 cm) was equilibrated with 50 mM phosphate buffer (pH 7.0). 33—35 mg protein was charged on to the column. The elution was done with the same buffer. The effluent was fractionated into 5 ml per tube with a flow rate of 7.5 ml per h. The absorbance of each effluent was read at both 280 and 405 nm and each enzyme activity was examined. V_0 represents the void volume estimated with Blue Dextran. (a), No elapsed time for storage at 4°C (immediately after the CM-cellulose chromatography was completed); (b) after 3 weeks' storage at 4°C (c) after 6 weeks' storage at 4°C. (See text for I, II, III and IV).

Fig. 2. Sedimentation pattern of the associated catalase molecules after 3 weeks' storage at 4°C. The photograph was taken 23 min after rotor reached full speed, 60 000 rev./min at 20°C. The enzyme concentration was 0.34% in 50 mM phosphate buffer (pH 7.0). Bar angle was 50°.

IV to component I increased (Fig. 1c). The ultracentrifugal analysis also revealed a pattern of multiple components having sedimentation coefficients of 11, 16 and 18 S after 3 weeks' storage at 4°C as shown in Fig. 2. Disc gel electrophoresis showed also the same profiles as the gel filtration and sedimentation behavior, as shown in Fig. 3. With elapsed storage time the proportion of the slower component to the native molecule increased, and this component may have a larger molecular size than the native, judging from disc gel electrophoretic behavior. The above-mentioned facts seem to indicate the molecular association of native enzyme during storage at 4°C. Also we noticed that the electrophoretic mobility of each component was always constant and reproducible, which is comparable to the result of gel filtration. Thus, the association of molecules was found to be time-dependent and to yield specifically the larger molecules.

Molecular weight of each component

The molecular weight of each component was estimated by both gel filtration and disc gel electrophoresis. From the result of disc gel electrophoresis, the molecular weights of components I, II and III were found to be about

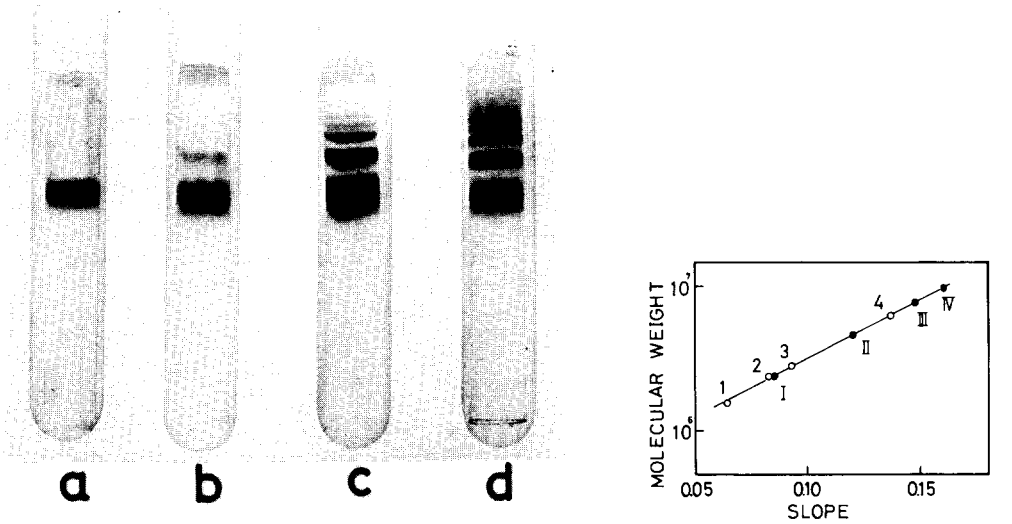


Fig. 3. Disc electrophoresis patterns of the associated molecules. Electrophoresis was carried out at room temperature for 120 min at 3 mA per tube. The protein was stained with 1.0% Amido Black. (a) no storage time (native enzyme); (b) after 1 week storage; (c) after 3 weeks' storage; (d) after 6 weeks' storage. The amount of proteins applied: (a) and (b), 63 μg; (c) and (d), 126 μg.

Fig. 4. Determination of molecular weight of the associated catalase molecules by polyacrylamide gel electrophoresis. 1, aldolase; 2, bovine liver catalase; 3, adenosinetriphosphatase from *B. stearothermophilus;* 4, glutamine synthetase from *S. cerevisiae.* Electrophoresis was carried out at room temperature for 90 min at 4 mA per tube. 40—50 μg of each standard protein was used. The abscissa is taken from the slopes shown in Fig. 5. I, component I (native monomer); II, component II (dimer); III, component III (trimer); IV, component IV (tetramer).

240 000, 480 000 and 750 000, respectively (Fig. 4). The molecular weight of the component IV was not exactly determined but presumed to be approximately one million. A similar result was obtained from gel filtration on a Bio-gel A-1.5 m column. From these results we concluded that the components II, III and IV are the dimer, trimer and tetramer, respectively of the native monomeric molecule.

Some properties of the associated molecules

Since the association of molecules was time-dependent, disc gel electrophoretic analysis of enzyme solution was performed at various times after storage at 4°C. Each gel was subjected to densitometric tracing analysis to estimate the relative amount of each component, which shows time-dependence of the association of molecules to form dimer, trimer, tetramer and molecules larger than tetramer (designated as polymers) (Table II). After 1 week storage, about 15% dimer was detected accompanying reduction of monomer (native molecule), while the dimer increased to about 22%, and 0.5% trimer and a trace of tetramer were detected after 2 weeks. After 6 weeks 4 components from monomer to tetramer were clearly observed and polymers were detected. It is noted that the monomer decreased gradually with elapsation of storage time, whereas the amount of dimer seemed to become almost constant after a long storage time. This fact may indicate formation of trimer and tetramer, and even polymers, through dimer as an intermediate of the associations.

TABLE II

TIME DEPENDENCE OF THE FORMATION OF THE ASSOCIATED MOLECULES DETECTED BY DENSITOMETRIC TRACING OF POLYACRYLAMIDE GELS

+, detectable; —, undetectable. The disc gel electrophoresis was carried out under the same condition as described in Fig. 3. After staining with Amido Black, the gels were subjected to densitometric tracing at 570 nm.

Time (day)	Component (%)				
	Monomer	Dimer	Trimer	Tetramer	Polymer
0	100	—	—	—	—
4	100	—	—	—	—
7	85.4	14.6	—	—	—
14	78.0	21.5	0.5	+	—
21	70.0	25.2	4.1	+	+
42	44.5	28.1	14.7	8.2	+

Fig. 5 shows a so-called Ferguson plot [15] of the associated catalase molecules (after 3 weeks' storage) on disc gel electrophoresis at varied gel concentrations. A plot of log R_m against gel concentration for catalase monomer, dimer, trimer and tetramer gave four non-parallel lines. The negative slopes of the lines increased as the association proceeded, which indicated the variation of sieving effect for different sized molecules when gel concentration was varied. Extrapolation of these lines gave a common convergence point where the gel concentration was about 8%. This fact indicates that the associated catalase molecules showed behavior of "size isomers" which have identical charges at a particular pH but different molecular sizes. This behavior was more evident in

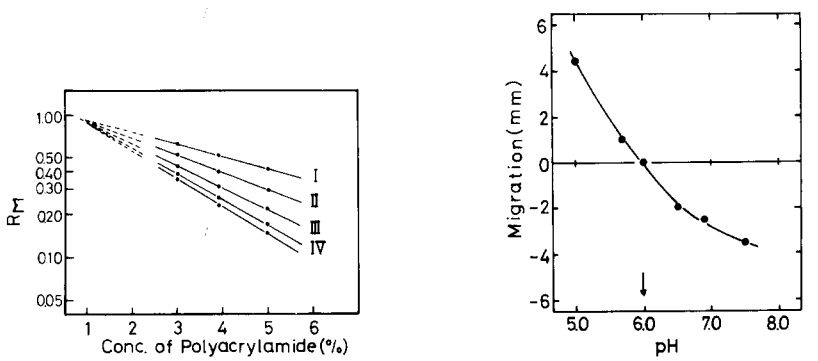


Fig. 5. The effect of different gel concentrations on the electrophoretic mobility of the associated catalase molecules. R_M represents the migration of protein relative to Bromphenol Blue. The concentration of polyacrylamide gel is represented in terms of percentage (w/v) of total monomer concentrations of both acrylamide and *N,N'*-methylenebisacrylamide, the latter being kept always at 0.4%. The conditions for electrophoresis were the same as those in Fig. 5. 230 μg protein were used. I, monomer (native); II, dimer; III, trimer; IV, tetramer.

Fig. 6. Determination of isoelectric point of porcine erythrocyte catalase by electrophoresis on cellulose acetate film (2 × 5 cm). The run was carried out at room temperature for 20 min at 2.5 mA and 160 V. The buffers used were sodium acetate buffer (pH 4.0—6.0, I = 0.1) and Tris/acetate buffer (pH 6.0—7.5, I = 0.1). After 3 weeks' storage of the enzyme solution at 4°C, the sample was subjected to electrophoresis.

experiments for estimation of isoelectric point. Fig. 6 illustrates the migration of the associated catalase molecules (after 3 weeks' storage) at various pH values on cellulose acetate film electrophoresis for determining the isoelectric point. Only a single band was detected on the film in the pH range examined, which is indicative of the "size isomer" nature of the associated molecules. The isoelectric point of the enzyme was 6.0.

Effect of thiol reagents on the association

In order to elucidate the cause of this specific association of the catalase molecules, a possible contribution of SH groups in the molecule was examined using some thiol reagents. It was found that addition of *p*-chloromercuribenzoate or monoiodoacetate to native monomer solution inhibited perfectly the association of molecules without any loss of enzymatic activity. The patterns of disc gel electrophoresis before and after addition of 1 mM *p*-chloromercuribenzoate to the monomer solution are shown in Fig. 7a. After the addition of *p*-chloromercuribenzoate only a single band was detected, though it was somewhat broadened. The same result was obtained by addition of 10 mM monoiodoacetate. These results strongly suggest that oxidation of SH groups to disulfide bond in catalase may cause the association of native molecules to form dimer, trimer, and tetramer etc., and the SH groups concerned may by unrelated to the enzymatic activity. Therefore, we attempted to convert the associated molecules into monomers by use of thiol reducing reagents such as 2-mercaptoethanol and dithiothreitol for reduction of disulfide bonds. The

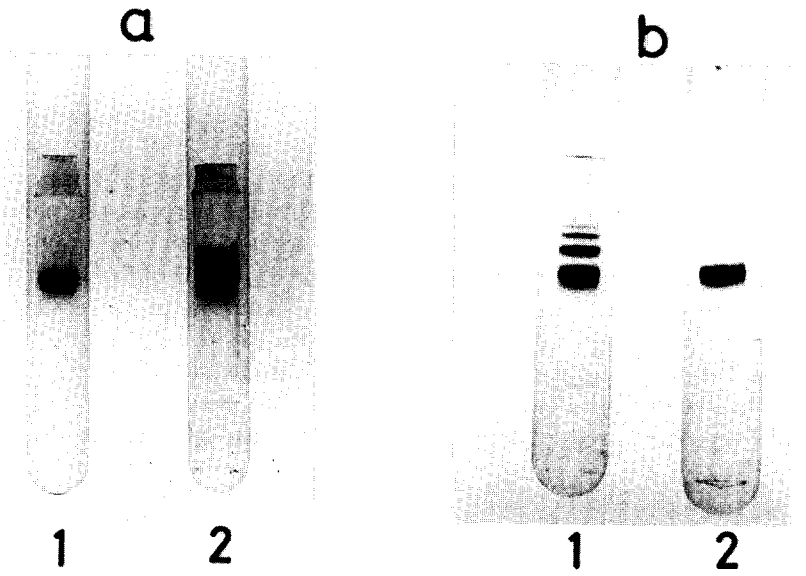


Fig. 7. Effect of thiol reagents on the association of catalase molecule detected by disc gel electrophoresis The electrophoretic run was performed under the same conditions as that in Fig. 3. (a) effect of addition of 1 mM *p*-chloromercuribenzoate to native catalase (monomer) solution. 1, before addition; 2, 3 weeks after addition of *p*-chloromercuribenzoate (stored at 4°C). (b) effect of addition of 10 mM 2-mercaptoethanol to the associated catalase solution (after 3 weeks' storage). 1, before addition; 2, 48 h after addition of 2-mercaptoethanol.

result on disc gel electrophoresis with 10 mM 2-mercaptoethanol is shown in Fig. 7b. The multiple bands seen in the associated enzyme solution (3 weeks' storage) were converted into a single band corresponding to the native monomer component after addition of the reagent. The same occurred after addition of 10 mM dithiothreitol, which was much more efficient for the reduction than 2-mercaptoethanol, since the former took only 3 h for complete conversion into monomers, whereas the latter needed 48 h. We also noticed that the Soret absorption spectra of the reduced monomers changed grossly; the Soret band at 405 nm was reduced to about half of the native level with blue shift to 400 nm, and a new hump appeared around 440 nm upon addition of 2-mercaptoethanol, whereas the Soret band decreased to about two-thirds with red shift to 410 nm by dithiothreitol. Furthermore, it should be pointed out that the reduced monomers completely lost enzymatic activity; the reason for this is now being investigated. Thus, SH groups were found to be involved directly in the association of catalase molecules, which may occur through the formation of disulfide bond by air oxidation.

Estimation of SH groups concerning the association of molecules

The dimer component was isolated in purity from other components by rechromatography of separated component II on a Bio-gel column after once passing through Bio-gel. Both monomer and dimer molecules were subjected to spectrophotometric titration to determine titratable free SH groups in each molecule. As a result, the numbers of free SH groups were estimated to be 8.2 and 8.3 for monomer and dimer, respectively. From this result it is inferred that 4 SH groups may be concerned with the association process from monomer to dimer. Thus, we can conclude that the association of catalase molecules is caused by air oxidation of SH groups to form disulfide cross-linkages between native molecules.

SDS gel electrophoresis of the associated molecules (3 weeks' storage) was carried out in the presence or absence of 2-mercaptoethanol. 8 M urea was added to the sample solutions containing 1% SDS in the presence or absence of 1% 2-mercaptoethanol and the mixture were kept overnight at room temperature prior to electrophoresis. In the presence of 2-mercaptoethanol only a single band was observed, having a molecular weight of about 70 000. On the other hand, in the absence of 2-mercaptoethanol three bands with molecular weights of 240 000, 120 000 and 60 000 were detected, which correspond to native, half-sized and 1/4-sized molecules, respectively. These results also indicate that the associated molecules are held together by disulfide bond and without 2-mercaptoethanol only partial reduction or disruption of disulfide bridges may occur.

Discussion

The present study revealed that native catalase molecule from porcine erythrocytes can be purified to a state both ultracentrifigally and electrophoretically homogeneous using DEAE-, CM-cellulose and Bio-gel column chromatography. However, the native molecule was converted into dimer, trimer, tetramer and the larger molecules with respect to native molecule (monomer), by

air oxidation of SH groups to form intermolecular disulfide bondings, upon storage at 4°C (Fig. 1). The molecular weights of dimer, trimer and tetramer were determined by both gel filtration and disc gel electrophoresis. The results obtained from gel filtration coincided well with those of disc gel electrophoresis, which gave values of about a half million, three quarters of a million and one million for dimer, trimer and tetramer, respectively, as compared with that of the native monomer, one quarter million. This association of the enzyme molecule showed time-dependence during storage; the polymerization proceeded with time upon storage as shown in Fig. 1 and Table II. It was also found that polymerization may occur through dimer from monomer as an intermediate. Nagahisa first succeeded in crystallizing porcine erythrocyte catalase with ammonium sulfate and found that the crystalline catalase sometimes showed two peaks upon sedimentation characterized by sedimentation coefficients of 11 and 15 S [1]. He claimed that the latter peak was due to the presence of a contamination persistently accompanying the genuine enzyme even after recrystallization. Regarding this phenomenon we inferred that the > 15-S component corresponds to the 16-S component found in our preparation (Fig. 2), which is considered to be dimer of the native molecule. Therefore, it may be concluded that this association of the native molecule occurs invariably when we prepare the purified sample in the atmosphere. These associated catalase moleculeas were found to be "size isomers" by disc gel electrophoresis and cellulose acetate film electrophoresis. The isoelectric point of the enzyme was 6.0, which is slightly higher than the value of 5.7 for bovine liver catalase [16].

When the SH groups of native monomer were blocked by *p*-chloromercuribenzoate or monoiodoacetate, no association of the molecule was detected with full enzyme activity even after 3 weeks (Fig. 7a). Furthermore, the addition of 2-mercaptoethanol or dithiothreitol to the solution of associated molecules caused the multiple bands on gel electrophoresis to reduce to only a single band corresponding to the monomer (Fig. 7b). We can deduce from these facts that the association is due to the oxidation of SH groups of the monomer to form disulfide bounds yielding dimer, trimer, tetramer and polymers, and that the SH groups may not be associated with enzyme activity. From spectrophotometric titration of SH groups with *p*-chloromercuribenzoate, four SH groups may be involved in the formation of dimer from monomer. Thus, the catalase molecule from porcine erythrocyte was found to associate specifically to form polymers by air oxidation of SH groups.

SDS gel electrophoresis measurement revealed the presence of a 1/4-sized component, which indicates that the native catalase may consist of four subunits [17—20]. A component having a molecular weight of 120 000 was detected in addition to two other components of 240 000 and 60 000 on the gel containing SDS in the absence of 2-mercaptoethanol. This component of 120 000 corresponds to a molecule half the size of the native one [21—23]. It may be inferred that the half-sized molecule may be either a splitting product of monomer or an associated molecule of two 1/4-sized molecules held together by some interactions.

No work has been reported so far on association or polymerization of erythrocyte catalase purified from various species. However, Mörikofer-Zwez

et al. [2] reported that human and horse erythrocyte catalase showed heterogeneity both in DEAE-cellulose chromatography and disc gel electrophoresis. They demonstrated that the catalase is separated into three fractions (A, B and C) on a DEAE-cellulose column, and that fraction B and C are derived from fraction A by oxidation of SH groups during preparation. Also they inferred that the transition of the chromatographic fraction A to fraction C is characterized either by a conformational change or by an irreversible oxidation of SH groups to higher oxidation products. Diezel et al. [24] reported aggregated forms of bovine liver catalase with molecular weights larger than that of native molecules, estimated by disc electrophoresis and density gradient centrifugation. Also they reported later that bovine liver catalase can be separated into several fractions with a uniform molecular weight of 240 000 by DEAE-cellulose chromatography and isoelectric focusing [25]. They also mentioned that these fractions showed different inclinations for dimerization of the native molecule during disc electrophoresis. The cause of the dimerization was uncertain but they claimed that the monomer molecules combined together noncovalently to form dimer. The specific association of porcine erythrocyte catalase found in the present study is essentially a different phenomenon from the aggregation or dimerization described above. Further detailed study on characterization of dimer of porcine erythrocyte catalase is now in progress.

Acknowledgement

The authors wish to thank Dr. A. Hachimori for his helpful discussions and Professor Y. Nosoh of Tokyo Institute of Technology for the ultracentrifugal analyses. The authors are also indebted to Professor Y. Kagawa and Dr. N. Sone of Jichi Medical School for use of the densitometer.

References

1 Nagahisa, M. (1962) J. Biochem. 51, 216—221
2 Mörikofer-Zwez, S., Cantz, M., Kaufman, H., Von Wartburg, J.P. and Aebi, H. (1969) Eur. J. Biochem. 11, 49—57
3 Aebi, H., Wyss, S.R., Scherz, B. and Skvaril, F. (1974) Eur. J. Biochem. 48, 137—145
4 Shirakawa, M. (1950) J. Agr. Chem. Soc. Japan 23, 361—362
5 Hachimori, A., Itoh, M. and Samejima, T. (1974) J. Biochem. 76, 1075—1081
6 Hachimori, A., Muramatsu, N. and Nosoh, Y. (1970) Biochim. Biophys. Acta 206, 426—437
7 Bonnichsen, R. (1955) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 2, pp. 781—784, Academic Press, New York
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
9 Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, Art 2, 321—349
10 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, Art 2, 404—427
11 Hedrick, J.L. and Smith, A. (1968) Arch. Biochem. Biophys. 126, 155—164
12 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
13 Summer, J.B. and Gralen, N. (1938) J. Biol. Chem. 125, 33—36
14 Boyer, P.D. (1954) J. Am. Chem. Soc. 76, 4331—4337
15 Ferguson, K.A. (1964) Metabolism 13, 985—1002
16 Summer, J.B. and Dounce, A.L. (1937) J. Biol. Chem. 121, 417—424
17 Sund, H., Weber, K. and Molbert, E. (1967) Eur. J. Biochem. 1, 400—410
18 Samejima, T., McCabe, W.J. and Yang, J.T. (1968) Arch. Biochem. Biophys. 127, 254—360
19 Furuta, H., Hachimori, A., Ohta, Y. and Samejima, T. (1974) J. Biochem. 76, 481—491
20 Takeda, A., Hachimori, A., Murai, M., Sato, K. and Samejiam, T. (1975) J. Biochem. 78, 911—924
21 Samejima, T., Kamata, M. and Shibata, K. (1962) J. Biochem. 51, 181—187
22 Samejima, T. and Yang, J.T. (1963) J. Biol. Chem. 238, 3256—3261
23 Deisseroth, A. and Dounce, A.L. (1970) Physiol. Rev. 50, 319—375
24 Diezel, W., Liebe, St., Kopperschläger, G. and Hofman, E. (1970) FEBS Lett. 6, 166—170
25 Diezel, W., Liebe, St., Kopperschläger, G. and Hofman, E. (1972) Acta Biol. Med. Germ. 28, 715—725