Biochimica et Biophysica Acta, 645 (1981) 71–80
Elsevier/North-Holland Biomedical Press

BBA 79302

SUPEROXIDE DISMUTASE WITHIN THE BOVINE ERYTHROCYTE MEMBRANE

A. PETKAU, T.P. COPPS and K. KELLY*

Medical Biophysics Branch, Whiteshell Nuclear Research Establishment, Atomic Energy of Canada Limited, Pinawa, Manitoba R0E 1L0 (Canada)

(Received October 8th, 1980)
(Revised manuscript received January 12th, 1981)

Key words: Superoxide dismutase; Asymmetry; (Bovine erythrocyte membrane)

(1) Membrane associated superoxide dismutase molecules in intact bovine erythrocytes were not readily accesible for reaction with rabbit ^{125}I-labelled antibodies, specific for the enzyme. Membrane alterations in ghost formation resulted in a 17-fold increase in uptake of ^{125}I-labelled antibody. The uptake of ^{125}I-labelled antibody by inside-out vesicles and intact ghosts was 3 : 1 in favour of the cytoplasmic surface, indicating an asymmetric distribution across the cell membrane. (2) Assay for enzyme activity after polyacrylamide gel electrophoresis of chloroform-ethanol extracts of two-day-old ghosts indicated the presence of 320 ± 105 mol of active enzyme per ghosts or 0.20 ± 0.07% of the cellular content. When examined over a 30-day storage period in-situ, the enzyme activity decreased by apparent first order kinetics to an equivalent of 110 ± 33 mol per ghost. The amount of protein extracted with chloroform-ethanol from ghosts and which electrophoresed with the same mobility as superoxide dismutase was in a large excess over enzyme protein, calculated from the measured activity, and increased with time of storage. Radioimmunoassay, however, detected no change in enzyme protein with storage. (3) The non-ionic detergent, Nonidet P-40, extracted twice as much active enzyme from ghosts as chloroform-ethanol. The detergent-extracted enzyme with the mobility of superoxide dismutase was resolved, using an immunosorbent of rabbit anti-superoxide dismutase antibody coupled to Sepharose, into an active component which bound to the immunosorbent and an inactive fraction, 0.23% of which could be restored to active superoxide dismutase by reconstitution with copper. The reactivated enzyme (2570 ng) was equal to 123% of the amount (2097 ng) detected by radioimmunoassay in the original membrane extract. However, this unique membrane component, whether in its active or reactivated form, could not be detected by the radioimmunoassay which employed antibodies directed against cytoplasmic superoxide dismutase.

Introduction

The erythrocyte membrane is frequently the focus of research investigating disease [1–10]. As well, the erythrocyte plasma membrane is frequently the model system with which to study structure-function relationships [11–20]. The activity of many enzymes in erythrocytes is confined to the plasma membrane while others are also found in the cytosol [21]. These enzymes are important to phosphorylation and dephosphorylation reactions, ion transport and nucleotide, carbohydrate and protein metabolism [21]. None of them, with the exception of the loosely bound glutathione peroxidase and glutathione reductase, are specifically suited to protect the plasma membrane against oxidative attack from superoxide radicals to which it is at risk from methemoglobin formation [22], thiol oxidation [23] and copper metabolism [24]. Since the plasma membrane appears sensitive to superoxide radicals [25], a requirement for superoxide dismutase within the

* Present address: Immunology Department, University of Manitoba, Winnipeg.

membrane is suggested. Indeed, by analogy to the higher peroxidase activity of membrane-bound catalase [26], superoxide dismutase within membranes may also be expected to be more active than in aqueous solutions. A recent study has indicated that human erythrocyte ghosts, prepared by hypotonic hemolysis, contained some, less than 0.3% of the superoxide dismutase originally present in the cells [27].

In this study, the reaction of membrane-associated superoxide dismutase, in situ, in the ghosts and inside-out vesicles of bovine erythrocyte membranes, with antibodies to the cytoplasmic bovine enzyme was examined. The change in the enzyme activity and enzyme protein with time of storage in situ in ghosts was also investigated. The enzyme was extracted both by chloroform-ethanol solvent and by treatment of the membranes with the non-ionic detergent, Nonidet P-40, and quantitated both by a double antibody solid phase radioimmunoaasay and by polyacrylamide gel electrophoresis for enzyme activity and enzyme-like protein. Finally, the enzyme extracted with Nonidet P-40 was resolved, using an immunosorbent of anti-superoxide dismutase antibody coupled to Sepharose, into an active component, which bound to the immunosorbent, and an inactive fraction which could be partially reactivated in terms of enzyme activity, yet remained unreactive with antibodies to cytoplasmic superoxide dismutase.

Materials and Methods

Ghost preparation

Fresh beef blood was collected from a local abattoir in 20 ml tubes containing 25.5 mg of the potassium salt of ethylenediaminetetraacetate (EDTA). The plasma and buffy coat were removed by centrifugation at $3\,000 \times g$ for 10 min and the red cells washed three times in isotonic saline. Red cell ghosts were prepared by successive hypotonic hemolysis; twice each in 60, 30, and 20 mosM sodium phosphate buffer pH 7.2 plus 1 mM EDTA, as previously described [28]. The residual hemoglobin in such preparations is less than 0.5% of the total protein in ghosts [28] and may be an integral part of the red cell membrane [29]. The number of ghosts/ml of sample was determined in a Coulter counter and checked in a hemocytometer. Inside-out vesicles of the ghosts were prepared as previously described [30] but in the absence of $MgSO_4$. The ghosts of some samples were fixed by Anderson critical point drying [31], shadowed lightly with gold in a Denton evaporator and examined for shape by scanning electron microscopy. With other samples, the superoxide dismutase activity and total protein were determined as previously described [32,33].

Comparative analyses for enzyme

Comparative analyses for superoxide dismutase from freshly prepared or stored bovine erythrocyte ghosts were done in parallel by radioimmunoassay [34] and by quantitative polyacrylamide gel electrophoresis [35]. For this purpose two parts (by volume) of ghosts were mixed with one part chloroform-ethanol (3 : 5, v/v) and stored in a refrigerator for 10 min. The mixture was then centrifuged at $8\,000 \times g$ for 4 min. The top layer was removed, and dried under a nitrogen stream. The residue was taken up in distilled water such that an overall concentration factor of six or greater was achieved.

When the non-ionic detergent, Nonidet P-40 (Particle Data Laboratories, Elmhurst, IL) was used to extract the membrane-bound enzyme, the detergent was first diluted to 10% with distilled water and then mixed 1 : 100 (by vol.) with suspensions of ghosts whose number typically was $9.9 \cdot 10^9$/ml. The mixtures were incubated at 37°C for 1 h following which they were centrifuged and the supernatant assayed for enzyme.

Extracts were analyzed by radioimmunoassay for superoxide dismutase activity and by polyacrylamide gel electrophoresis [35] for both enzyme activity and protein. A typical electrophoresis run consisted of 12 cylindrical gels, seven of which were used for variable amounts (1–40 μl) of our reference enzyme solution (50 ng/μl of bovine erythrocyte superoxide dismutase from Dr. J.V. Bannister, Malta). The remaining five gels were layered with variable volumes (5–80 μl) of an extract. After electrophoresis [35], the individual gels were stained for enzyme activity [35] and scanned for optical transmittance at 580 nm in an Isco Gel Scanner Model 1310. The change in transmittance within the two bands associated with the enzyme was recorded on chart paper and the areas corresponding to the bands integrated by planimetry. Per cent transmittance increased with log

(amount of standard enzyme solution) and the slope of the line was used to calculate the amount of active enzyme in each volume of extract run. These results were used to calculated the number of active enzyme molecules per erythrocyte ghost. In a similar fashion, the amount of protein in extracts, electrophoresing as superoxide dismutase was determined, except that absorbance at 650 nm, rather than transmittance, was used to scan the protein-stained gels [35]. In this case, the integrated areas corresponding to cupro-zinc superoxide dismutase in the standard solution increased linearly with the amount applied.

Preparation and isolation of rabbit antibodies to bovine superoxide dismutase

Outbred white rabbits were immunized at monthly intervals, with 2 mg superoxide dismutase in Freund's complete adjuvant. The sera were tested for antibody content and specificity in a double antibody solid phase radioimmunoassay, as previously described [34].

Antibodies were isolated from pooled sera by affinity chromatography. An immunosorbent was prepared by first coupling 50 mg normal rabbit IgG to 50 ml cyanogen bromide-activated Sepharose 4B [36]. Superoxide dismutase was then coupled to the IgG on the Sepharose using a two-step glutaraldehyde activation process [37]. The Sepharose-IgG was activated with glutaraladehyde, preheated at 50°C for 30 min, at a final concentration of 1% in phosphate buffered saline (pH 7.4), After 1 h at room temperature, the gel was filtered and washed exhaustively with phosphate buffered saline, then stirred for 3 h at room temperature with 50 ml of a 1 mg/ml solution of superoxide dismutase in phosphate buffered saline. Excess glutaraldehyde-activated groups were neutralized by incubation of the immunosorbent in 2 M NH_2OH (pH 7.4). The pooled serum was incubated at 4°C for 16 h with the immunosorbent, Sepharose-IgG-superoxide dismutase, which was subsequently filtered and washed exhaustively on a Buchner funnel with 0.5% Tween 20 in phosphate buffered saline to remove nonspecifically bound protein. Adsorbed antibodies were then eluted by treatment with 4 M guanidine hydrochloride (Schwarz/Mann Ultra Pure Reagent, Orangeburg, NY) in 0.25 M sodium acetate buffer (pH 4.0). The antibody preparation was then dialysed against 0.05 M Na_2CO_3-$NaHCO_3$ buffer (pH 10) for conjugation to Sepharose 4B. 25 ml Sepharose 4B (packed volume at $800 \times g$) was activated with 1 g cyanogen bromide [36], then reacted with 40 mg rabbit antibody in 25 ml 0.05 M Na_2CO_3-$NaHCO_3$ buffer (pH 10) for 16 h at 4°C with gentle shaking. The immunosorbent, Sepharose-rabbit anti-superoxide dismutase antibody, was then treated with 0.5 M ethanolamine (pH 10) for 3 h at room temperature to block any remaining reactive sites on the gel, followed by 4 M guanidine hydrochloride (pH 4.0) for 15 min at room temperature to remove noncovalently bound protein, and finally washed and equilibrated in 0.5% Tween 20 in phosphate buffered saline (pH 7.4).

Rabbit anti-superoxide dismutase antibody (500 μg), normal IgG (500 μg) and superoxide dismutase (50 μg) were radioactively labelled with ^{125}I (Amersham-Searle, Arlington Heights, IL) by the chloramine-T method [38]; the free iodine was removed by filtration through BioGel P-60, as described previously [35]. The specific activities of the ^{125}I-labelled antibody and normal IgG are given in Table I per μg protein; for ^{125}I-labelled superoxide dismutase it was $9.5 \cdot 10^6$ cpm/μg.

Affinity chromatography of Nonidet P-40 extracts

Preliminary trials indicated that treatment of ghosts with 0.1% Nonidet P-40 extracted 3.5 to 3.8 times more protein and 2.0 ± 0.4 times more active enzyme than chloroform-ethanol. Therefore, 0.38 ml of 10% Nonidet P-40 solution in distilled water was incubated for 1 h at 37°C with 38 ml of freshly prepared bovine erythrocyte ghosts. The extract solution was clarified by centrifugation at $1\,000 \times g$ for 15 min and concentrated in an Amicon 52 stirred cell with a PM 10 membrane (>10 000 mol.wt. cutoff) to approx. 10 ml. Analyses by radioimmunoassay detected no enzyme present in the fitrate from the Amicon. Freshly prepared ^{125}I-labelled superoxide dismutase (approx. 170 000 cpm/0.2 ml) was added as a marker to the Amicon concentrate, designated as S1. The concentrate was too viscous and was centrifuged at $42\,000 \times g$ for 45 min to remove particulate material. The supernatant, S2, was then subjected to affinity chromatography.

A column (1.1 × 5.3 cm, 5 ml) of Sepharose-rabbit anti-superoxide dismutase antibody was treated with 4 ml of 4 M guanidine hydrochloride (pH 4.0) and

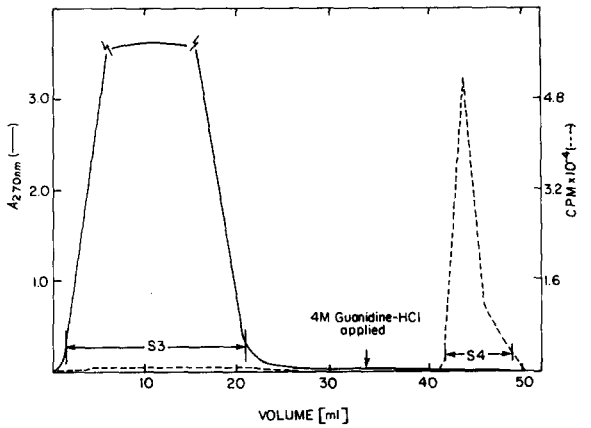

Fig. 1. Separation of active (sample S4) from inactive (sample S3) superoxide dismutase from bovine red cell ghost membranes by affinity chromatography on Sepharose antibody column. Active enzyme, reactive with rabbit antibodies to bovine superoxide dismutase was eluted with 4 M guanidine hydrochloride, pH 3.8. Sample S3 contained reactivatable enzyme plus other protein.

equilibrated in phosphate buffered saline (pH 7.4) containing 0.5% Tween 20. Sample S2 (15.4 ml) was passed through the column at a flow rate of 15 ml/h. The column was then washed with phosphate buffered saline till the A_{270nm} had fallen to the background level of ≤ 0.02. A volume of 4 ml of 4 M guanidie hydrochloride (pH 4.0) was then applied to the column, followed by phosphate-buffered saline. The flow rate was maintained at 15 ml/h throughout. The effluent was collected in 1.4 ml fractions and both the A_{270nm} and radioactivity measured. The elution profile is shown in Fig. 1. Protein not retained by the immunosorbent is designated as S3 while the guanidine hydrochloride eluate is designated as S4. Both S3 and S4 were dialysed against phosphate-buffered saline for 48 h before analysis.

Radioimmunoassay

Bovine superoxide dismutase in aqueous solution was quantitated by a double antibody solid phase radioimmunoassay, the development of which has been described previously [34]. Enzyme is quantitated by the extent of inhibition of the binding of ^{125}I-labelled superoxide dismutase by antibody.

Briefly, volumes of 0.1 ml of rabbit antiserum at a dilution of 1 : 2000 in normal rabbit IgG at 100 μg/ml in assay buffer, were reacted for 3 h at 37°C with 0.1 ml of serial 2-fold dilutions of samples containing the enzyme of unknown concentration. ^{125}I-labelled superoxide dismutase (approx. 70000 cpm/0.1 ml) was next added and the mixture reacted for 16 h at room temperature. Enzyme bound to rabbit antibody was separated from free enzyme by the subsequent reaction for 3 h at 37°C with 0.15 ml of a suspension in assay buffer of sheep anti-(rabbit IgG) antibody coupled to Sepharose 4B. The sepharose solid phase was washed three times with 1 ml of cold assay buffer before measuring the bound radioactivity (B^*). A 100-fold excess of superoxide dismutase to ^{125}I-labelled superoxide dismutase was added as inhibitor to determine the amount of radioactivity (C) bound nonspecifically to the solid phase. Assay buffer was added in lieu of enzyme to determine B_0^*, i.e. cpm ^{125}I-labelled superoxide dismutase bound in the absence of inhibitor. All assays were done in duplicate and included the reference standard. Inhibition curves were prepared by plotting $(B^* - C)/(B_0^* - C)$ against the logarithm$_{10}$ of the reciprocal of the dilution factor for samples containing unknown amounts of enzyme.

Results

The bovine superoxide dismutase that has been used throughout this study for the production and isolation of rabbit antibodies and as a reference standard in radioimmunoassay and polyacrylamide gel electrophoresis, has been extracted from the cytoplasm of erythrocytes in the laboratory of Dr. J.V. Bannister. It has been amino acid sequenced (personal communication from Dr. Bannister), is of recognized purity in electrochemical studies [39], is the most stable by electrophoretic analysis, has the highest specificity activity and is radiobiologically the most effective [35] in comparison with other commercially available preparations.

The inact red cells showed no significant uptake of ^{125}I-labelled immunoglobulin to the outer cell surface (Table I). However, the uptake of rabbit ^{125}I-labelled antibody by the ghosts increased by a factor of 17 to 25 ± 13 antibody molecules/cell. This increased accessibility of membrane enzyme to reaction with antibody is evidently the result of changes in the composition and other properties of the membrane in the formation of ghosts [40,41].

TABLE I

COMPARATIVE UPTAKE OF ^{125}I-LABELLED RABBIT ANTIBODY, SPECIFIC TO CYTOPLASMIC SUPEROXIDE DISMUTASE FROM BOVINE ERYTHROCYTES, AND ^{125}I-LABELLED NORMAL IMMUNOGLOBULIN (IgG) BY BOVINE RED CELL MEMBRANE PREPARATIONS

Nature of membrane	^{125}I-labelled immunoglobulin uptake			Protein (% input)	Net uptake of ^{125}I-labelled antibody (mol/cell) (Mean ± σ)
	(i) ^{125}I-labelled antibody (cpm/cell) × 10^{-5} (Mean ± σ)	(ii) ^{125}I-labelled normal IgG (cpm/cell) × 10^{-5} (Mean ± σ)	Ratio (i/ii) *		
Washed red cells	0.52 ± 0.04	0.46 ± 0.05	1.1	–	<1
Ghosts	3.3 ± 1.6	0.19 ± 0.07	17	100	25 ± 13
Top band of inside-out vesicles	2.0 ± 0.4	0.14 ± 0.03	14	20	75 ± 15 **

* Corrected for the difference in specific activity of ^{125}I-labelled antibody ($4.7 \cdot 10^6$ cpm/μg) and normal IgG ($4.5 \cdot 10^6$ cpm/μg).

** Normalized to 100% protein (fifth column).

Nonetheless, scanning electron microscopy of the ghosts showed the characteristic concave surface of red cells in contrast to the more spherical appearance of glutaraldehyde-fixed ghosts [42] and the suggestion that hypotonic hemolysis distorts and fragments red cell membranes [43]. The greater loss of shape observed by these workers may possibly result from glutaraldehyde fixation and is avoided by Anderson critical point drying.

Inside-out vesicle formation obviously requires marked alterations to the shape of erythrocyte ghosts [30] and these changes were accompanied by release of protein into the supernatant. As isolated inside-out vesicles are depleted of spectrin [44], it may be concluded that much of this protein is spectrin [30,44], a major protein selectively associated with the cytoplasmic side of erythrocyte ghosts [28]. This depletion plus the possible loss of other proteins was not accompanied by a proportionate decrease in superoxide dismutase. On the contrary, the net uptake of ^{125}I-labelled antibody by inside-out vesicles increased to 75 ± 15 mol/cell when normalized to 100% protein input (Table I). This enhanced uptake of enzyme-specific antibodies by the inside-out vesicles, whose extrinsic proteins on the cytoplasmic surface have been removed to an increased extent, indicates that the enzyme molecules are intimately associated with the inner surface of erythrocyte ghost membranes. As well, the 3-fold greater uptake of enzyme-specific antibodies by inside-out vesicles suggests that the enzyme is either more populous, accessible or reactive on the cytoplasmic side of red cell membranes than is the case with their outer surface. Neither ghosts nor inside-out vesicles showed an increase in uptake of ^{125}I-labelled normal IgG over that of intact erythrocytes (Table I, third column).

The total protein in the erythrocyte ghost membrane by the method of Lowry et al. was $(0.37 \pm 0.01) \cdot 10^{-12}$ g/ghost. The same value was obtained on Nonidet P-40 extracts. By contrast, the chloroform-ethanol solvent extracted only $(0.09 \pm 0.02) \cdot 10^{-12}$ g of protein per ghost or 3.8-times less. The former value is consistent with those from Brain [13] and Dodge et al. [45].

Assay for superoxide dismutase activity in the chloroform-ethanol extracts by the nitro blue tetrazolium technique [32] gave biphasic inhibitory-stimulatory curves and therefore the results were considered unreliable. Alternative techniques were developed with which to assay the enzyme in red blood cell membranes.

The results of the alternative measurements, based on radioimmunoassay and quantitative polyacrylamide gel electrophoresis of chloroform-ethanol (3 : 5, v/v) extracts, are given in Fig. 2. In 2-day-old ghosts, quantitative electrophoresis indicated the presence of 320 ± 105 mol/red cell membrane or 0.2 ± 0.07% of the cellular content. This value dropped monotonically to 100 ± 33 mol/cell if the membrane-bound enzyme was left in situ and the ghosts

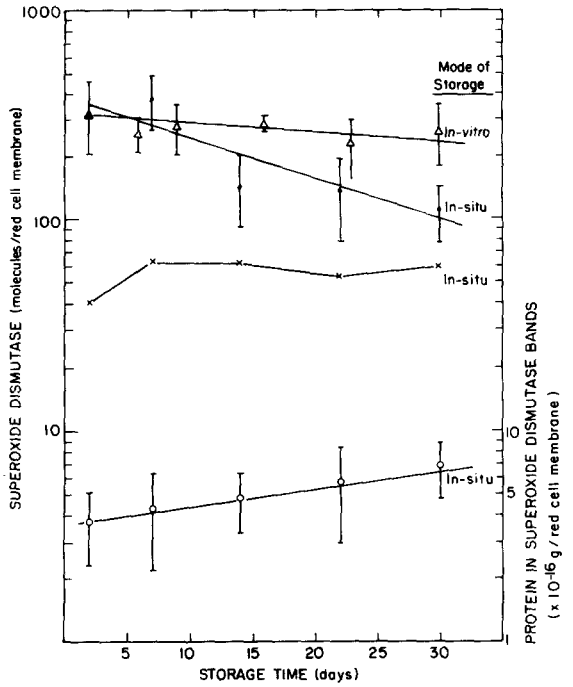

Fig. 2. Left hand ordinate: Number of active superoxide dismutase molecules per bovine red cell membrane vs. storage time in vitro (△) and in situ (●,X). Enzyme activity measured by radioimmunoassay (X) and by quantitative polyacrylamide gel electrophoresis (△,●). Right hand ordinate: Protein in superoxide dismutase electrophoretic bands of bovine red cell ghost membranes vs. storage time in situ (○). Enzyme and protein extracted with chloroform/ethanol (3 : 5, v/v).

were stored with refrigeration for 30 days. On the contrary, if the membrane-bound enzyme was extracted with chloroform-ethanol from the 2-day-old ghosts and then stored in vitro with refrigeration for 30 days, the enzyme activity decreased relatively slightly to 265 ± 88 mol/red cell membrane. Taken together, these results indicate that the membrane-bound enzyme is inactivated by interacting chemically with ghost membrane constituents. Kinetically, the inactivation process is consistent with a first order reaction, the nature of which remains to be identified.

The radioimmunoassay on chloroform-ethanol extracts yielded an average value of 56 ± 9 enzyme molecules per red cell membrane which did not vary with time of storage in situ or in vitro (Fig. 2). This value is 5.7-times less than that determined by quantitative electrophoresis on extracts of 2-day-old ghosts. Radioimmunoassay on Nonidet P-40 extracts yielded an average value of 118 ± 14 enzyme molecules per membrane indicating that extraction with detergent was more efficient than the chloroform-ethanol method for isolation of superoxide dismutase from membranes.

Also in Fig. 2 is plotted, with time of storage in situ, the amount of membrane-protein extracted with chloroform-ethanol which migrated with the same mobility as superoxide dismutase in quantitative gel electrophoresis. On day 2, this amounted to $3.7 \cdot 10^{-16}$ g per cell membrane which, if it were all superoxide dismutase, would be equivalent to approx. 6 900 mol or 4% of the cellular content. This increased to a level of $6.7 \cdot 10^{-16}$ g per cell membrane over a 30-day period. The discrepancy in the amounts of enzyme associated with bands with the mobility of superoxide dismutase, based on enzyme activity (320 mol per cell membrane) and protein (approx. 6 900 mol per cell membrane) measurements, suggests either that a large fraction consists of protein which is not like superoxide dismutase in activity but possesses similar physical properties or that a large fraction of the enzyme is in an inactive form.

Affinity chromatography of membrane extracts

Radioimmunoassay on the concentrated lysate S1, extracted with 0.1% Nonidet P-40, yielded an enzyme content of 2 097 ng (Table II). High speed centrifugation (42 000 × *g* for 45 min) of S1 sedimented only 9% of the radioactive ^{125}I-labelled superoxide dismutase marker that was added to S1. Radioimmunoassay revealed, however, that only 47% of the enzyme in S1 remained in the supernatant S2 (Table II). Passage of S2 through the column of Sepharose-rabbit anti-superoxide dismutase antibody removed all enzyme detectable by radioimmunoassay. The unabsorbed effluent S3 contained only 4% of the radioactivity and less than 3 ng superoxide dismutase per ml, which is the sensitivity of the radioimmunoassay (Fig. 3). Treatment of the column with 4 M guanidine hydrochloride (pH 4.0) eluted 82% of the ^{125}I-labelled enzyme that had been bound to the antibody. But radioimmunoassay on the eluate S_4 revealed only a 17% recovery of the amount applied in S2. Most likely this is the result of an irreversible unfolding effect of guanidine hydrochloride on the conformation of superoxide dismutase, affecting its

TABLE II

SEPARATION BY AFFINITY COLUMN CHROMATOGRAPHY, USING SEPHAROSE-RABBIT ANTI-SUPEROXIDE DISMUTASE ANTIBODY, OF ACTIVE AND INACTIVE SUPEROXIDE DISMUTASE FROM BOVINE RED CELL MEMBRANES

Sample	Radioimmunoassay for superoxide dismutase in red cell membrane fractions [a]			
	ng/ml	ml	Total (ng)	Remark
Filtrate of concentrated red cell membrane lysate (S1)	233	9	2097 [b]	
S1 supernatant (S2)	64	15.4	986	47% of S1
Affinity column effluent (S3)	n.d.	19.4	(–) [c]	
Affinity column eluate (S4)	86	4	172	17% of S2

[a] Extracted with Nonidet P-40.

[b] Equivalent to approx. 105 mol of enzyme per red cell membrane.

[c] 2570 ng reactivated to active superoxide dismutase after copper reconstitution. n.d., not detectable (<3 ng).

reactivity with antibody in the radioimmunoassay. Other eluting agents which will provide for greater recovery of active enzyme are presently being examined.

Sample S3 contained 8.0 ± 2.2 mg protein per ml and resolved into 14 bands on polyacrylamide gel electrophoresis. No active enzyme could be detected in samples of S3, concentrated further by a factor of 3.13, on electrophoresis. Aliquots of S3 were checked for copper and zinc by atomic absorption [46] before and after dialysis against 100 μM $CuSO_4$ for 24 h and then distilled water for 3 days. Before dialysis against $CuSO_4$ its copper and zinc content were 21 and 8%, respectively, of that in an equal weight of superoxide dismutase. After dialysis, the zinc content remained unchanged but the copper rose to 492% of an equal weight of enzyme. Apparently, superoxide dismutase is not the only membrane moiety which takes up copper, in agreement with other observations [47]. The uptake of copper reactivated 2 570 ng of enzyme in the S3 sample, as measured by enzyme activity in bands with the the mobility of superoxide dismutase in quantitative electrophoresis. This represents a ((2 570/2 097) × 100 =) 123% increase over that initially present in the concentrated lysate, S1, of red cell membranes. Radioimmunoassay on reactivated S3 sample, however, failed to detect any enzyme although the concentration (>100 ng/ml) of enzyme greatly exceeded the minimum concentration detectable by radioimmunoassay. One can only conclude that the reactivated enzyme is antigenically unrecognizable by antibodies raised against cytoplasmic superoxide dimutase. Moreover, the 2 570 ng of enzyme reactivated through copper replacement represents only 0.23% of the protein associated with the two electrophoretic bands of the cupro-zinc enzyme. Conceivably, more of this protein can be reactivated by other means, a task that is outside the scope of the present study.

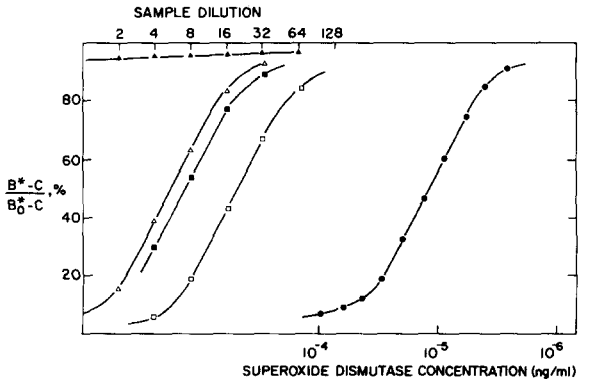

Fig. 3. Radioimmunoassay inhibition curves where $(B^* - C)/(B_0^* - C)$ is plotted against the reference superoxide dismutase concentration (ng/ml) (•), and against the serial 2-fold dilutions of the samples S1 (□), S2 (△), S3 (▲) and S4 (■) from the affinity chromatography of membrane extract.

Discussion

Before one discusses the significance of the data obtained on superoxide dismutase associated with the

membranes of bovine erythrocytes, it is necessary to discuss first the methods used for quantitation of the enzyme, particularly in membrane extracts. Subjection of ghosts to disruption by chloroform-ethanol or to Nonidet P-40 detergent would not necessarily release the same amount of the enzyme in its native configuration. In addition, the enzyme may still be associated with lipid from the membrane to a varied extent. Thirdly, subjection to chloroform-ethanol or detergent would affect the conformation and also the specific activity of the enzyme. Both the polyacrylamide gel electrophoresis and radioimmunoassay methods used in this study attempt to quantitate membrane associated enzyme by comparison with known concentrations of superoxide dismutase extracted with chloroform-ethanol from the cytoplasm of bovine erythrocytes.

Quantitative gel electrophoresis measures the enzyme activity per unit protein which has the same mobility as the reference standard. The uptake of Cu^{2+} reactivated 2 570 ng of enzyme in the S3 membrane fraction, but this amounts to only 0.23% of the protein within the two electrophoretic bands of the cupro-zinc enzyme. It is conceivable that the remaining protein is non-enzyme membrane components with the same mobility as the standard or it may contain some fraction of inactive enzyme. This method of assay is incapable of distinguishing between the two.

The radioimmunoassay quantitatives superoxide dismutase by the extent of inhibition of binding of ^{125}I-labelled superoxide dismutase by specific rabbit antibody. Accurate quantitation required that the enzyme in test samples have the same antigenicity and therefore react with equal affinity with antibody as the reference standard. The concentration is obtained by relating the extent of inhibition to a known concentration of an equivalent standard solution. Antigenic identity and hence affinity for antibody are examined by testing the inhibition curves of the test samples and standard for parallelism. Lack of parallelism proves dissimilarity between the inhibitors in the unknown and standard solutions [48]. Lack of parallelism was not observed (Fig. 3). The antibodies used have been shown to be monospecific for cytoplasmic bovine superoxide dismutase with no cross reaction with murine or human enzyme. When the uptake of active superoxide dismutase (from bovine erythrocytes) by murine lymphocytes was measured by both quantitative electrophoresis and radioimmunoassay, comparable values of 17–19% were obtained [34]. It is hardly likely, therefore, that a systematic error in the procedures was responsible for the discrepancy between the two sets of results. One can visualize that Nonidet P-40 could affect the conformation and hence antigenicity of the enzyme differently from chloroform-ethanol. However, inhibition curves by enzyme in both detergent and chloroform-ethanol extracts were parallel. Radioimmunoassay could underestimate the enzyme content of samples if association with lipid or other membrane protein blocks the reaction of antibody with the enzyme or if there were enzymatically active membrane components which were antigenically distinct from the cytoplasmic superoxide dismutase.

The gel-based assay for active superoxide dismutase per bovine ghost on chloroform-ethanol extracts gave $0.2 \pm 0.07\%$ of the cellular inventory which is in good agreement with an earlier measurement of $<0.3\%$ on human red cells ghosts [27]. On Nonidet P-40 extracted ghosts, however, our value was increased by 2.0 ± 0.4 times to $0.4 \pm 0.2\%$. In comparison, the radioimmunoassay of chloroform-ethanol and Nonidet P-40 extracts of bovine ghosts gave values of 0.04 ± 0.006 and $0.07 \pm 0.008\%$, respectively. These data suggest, therefore, that a large fraction of active enzyme in the membrane extracts is antigenically distinct from the cytoplasmic enzyme. However, the existence of antigenically similar superoxide dismutase molecules embedded in the membrane is evident from the uptake of rabbit ^{125}I-labelled antibodies specific to the cytoplasmic enzyme. These enzyme molecules are clearly not readily accessible in intact cells (Table I). However, membrane alterations occurring through ghost formation and in the preparation of inside-out vesicles appear to expose more enzyme to reaction with antibody.

The uptake of ^{125}I-labelled antibody by intact ghosts and inside-out vesicles was 3 : 1 in favour of the cytoplasmic surface. This asymmetry in distribution is not unexpected in view of the enzyme's cytoplasmic origin [49] and the resulting outward movement down a concentration gradient. Within the red cell membrane, the negatively charged enzyme would be subjected to an appropriately-directed electric field, derived from the transmembrane potential

[50], which should aid its movement to the membrane's outer surface. Since the apoenzyme, partially or wholly depleted in coordinated metal ions, has the greater electrophoretic mobility and a greater affinity for lipid bilayers, it may well localize preferentially toward the outer surface of the red cell membrane. The utilitarian value of these intramembrane electrophoretic processes is best appreciated by considering, on the one hand, the protective role of active superoxide dismutase within membranes and, on the other, the possible need for the red cell membrane to divest itself of the enzyme when inactive.

The results in Fig. 2 clearly show that the activity of superoxide dismutase in ghost membranes decreases with storage time. The inactivation process, which conceivably could involve lipid peroxidation, apparently produced no detectable changes in antigenic conformation as the enzyme content (56 ± 9 mol per membrane, as determined by radioimmunoassay) remained constant with time of storage in situ.

Affinity chromatography on Nonidet P-40 extracts of bovine red cell ghosts clearly demonstrated that the membrane extracts contained an active form of enzyme which appears to be antigenically similar to the cytoplasmic superoxide dismutase. This form behaved similarly to the ^{125}I-labelled cytoplasmic enzyme, added as a marker to the extract, in its reactivity with the insolubilized antibody on the column. Radioimmunoassay confirmed that this active form was quantitatively absorbed out by the immunosorbent. Yet there remained within the extract a considerable amount of protein which had the same mobility as superoxide dismutase, 0.23% of which could be reactivated by restoration of Cu^{2+}, and which, in both its inactive and reactivated forms, was completely unreactive with antibody.

This raises quanstions as to the nature of this membrane associated enzyme protein which is antigenically distinct from the cytoplasmic enzyme. Is it cytoplasmic superoxide dismutase which has lost its reactivity with antibody through association with membrane components or did it lose its antigenicity when it became inactive? Or has is always been an antigenically distinct form of superoxide dismutase unique to the red cell membrane? The answers to these questions hopefully will be obtained when antibodies specific to this unique membrane component are produced.

Acknowledgements

The technical assistance of R.A. Zepp, W.S. Chelack, H. Boux and P. Impey is gratefully acknowledged.

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