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New evidence for the essential role of arginine residues in anion transport across the red blood cell membrane *

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2,3-Butanedione, in the dark and in the presence of borate, reacts rapidly to inactivate the sulfate equilibrium exchange across the human red cell membrane. Reactivation occurs spontaneously after the removal of borate, indicating the reaction of butanedione with essential arginine residues. The inactivation of the transport system depends on the concentration of the reagent, on the incubation time and exhibits pseudo-first-order kinetics. Chloride ions are able to protect the transport system against inactivation with the reagent. This would suggest the participation of the modified residue in the substrate binding site. When the transport system is inhibited to 50–60% by butanedione, the transporter can still bind covalently the anion transport inhibitor $^2H_2$DIDS up to 85 ± 12% of its total binding capacity. $^3H_2$DIDS concentration was either 3.15, 10 or 20 μM. Modification of resealed ghosts with 50 mM butanedione under conditions where the transport system is to more than 75% inhibited, causes a reduction of only about 30% of the reversibly bound $^3H_2$DIDS.

Introduction

Anion exchange across the plasma membrane of the human red cell is catalyzed by a 97 kDa integral membrane protein [2,3]. This protein is known as band 3 [4]. It was reported earlier from this laboratory that 1,2-cyclohexanedione and phenylglyoxal were found to be potent inhibitors of sulfate equilibrium exchange across the red blood cell membrane (Zaki [5–10]. Wieth et al. [11] also found that chloride exchange in red cells can be inactivated by phenylglyoxal. These components are known for their specificity for arginine residues in proteins. The probability that these reagents could have their inhibitory effect by reacting with other amino acid side chains rather than arginine could not be totally excluded [12]. Since quantitation of arginine modification with these compounds can only be done through amino acid analysis by measuring the loss of arginine, this procedure is not capable of detecting changes of one or a very few residues in proteins that contain a large number of arginine residues. For this reason we have not been able to measure the difference in the arginine content of the modified and unmodified band 3 protein. Since band 3 contains about 44 arginine residue, and complete

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Abbreviations: H_2DIDS 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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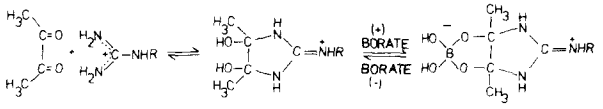
inhibition of the anion transport with phenylglyoxal is accompanied by modification of 2 to 3 arginine residues [9]. In order to added more evidence for the essential role of arginine residues in the process of anion transport across the red blood cell membrane, another reagent, 2,3-butanedione has been used. The use of monomeric 2,3-butanedione in borate buffer as a highly selective reagent for modification of arginine residues in the dark has been introduced by Riordan and co-workers [13]. The initial reaction between butanedione and a guanidinium group involves the formation of two N-H bonds across the carbonyl group of butanedione. The adduct has vicinal hydroxyl groups. If they are in the *cis* configuration the adduct can be stabilized by borate. It is unlikely that such stabilization occurs upon reaction with other amino acids.



Scheme I.

The reaction scheme, represents the reaction of butanedione with guanidinium moiety of the arginine residue according to Riordan [14] and Yankeelov [15]. Butanedione reacts reversibly with the guanidino group of arginine to form the *cis*-diol dihydroxyimidazoline derivative which then complexes rapidly and reversibly with borate. Thus, in the presence of borate the reaction proceeds faster due to product stabilization. This reaction scheme shows the role of borate in the stabilization of the dicabrinolamine (4,5-dimethyl-4,5-dihydroxy-2-imidazoline) adduct.

Materials and Methods

All experiments were performed with human erythrocytes from healthy donors. Blood was obtained from the Red Cross in Frankfurt/Main and stored at 4° C in acid/citrate/dextrose buffer for 2–4 days. After removal of plasma and buffy coat the cells were washed three times in isotonic buffer. Resealed ghosts were prepared essentially as in Ref. 1. The cells were hemolyzed at 0° C at a cell to medium ratio of 1 : 20 in a medium containing 4 mM $MgSO_4$ and 1.45 mM acetic acid. 5 min after hemolysis sucrose, gluconate, citrate and Hepes were added from a concentrated stock solution to obtain, a final concentration of 200 mM sucrose, 27 mM gluconate, 25 mM citrate and 5 mM Hepes in the hemolysate. After centrifugation at 0° C the ghosts were resuspended and resealed in standard buffer containing the same concentration of sucrose, gluconate, citrate and Hepes are as above, in addition, 1 mM Na_2SO_4 was added.

Modification of resealed ghosts with 2,3-butanedione. The reaction of the resealed ghosts with 2,3-butanedione was carried out in the dark at 37° C, either in a standard buffer containing 50 mM borate, 25 mM citrate, 27 mM gluconate, 5 mM Hepes and 1 mM Na_2SO_4 or in standard buffer without borate. The inhibition of SO_4^{2-} exchange was measured either at pH 8.0 or pH 7.4. The reaction time and the concentration of the 2,3-butanedione are indicated in the figure legends. $^{35}SO_4^{2-}$ equilibrium exchange was measured after removal of excess of butanedione by washing five times in a medium which had the same composition as that used for the reaction in presence or absence of borate. Two washes contain 0.5% bovine serum albumin (crystallin, Serva). The flux measurements were done as described previously [2]. Transport is expressed as a percent of the residual activity relative to a control value in the same media used for the reaction either is presence or absence of borate but without the inhibitor. The kinetic data were fitted with a least squares method by nonlinear regression.

Chemicals. 2,3-Butanedione was obtained by Aldrich-Chemie, Steinheim. Hepes was obtained from Carl Roth KG, Karlsruhe. All other substances were purchased from Merck-Darmstadt.

Results and Discussion

Inactivation of sulfate equilibrium exchange in resealed ghosts with 2,3-butanedione in presence of borate ions

The incubation of resealed ghosts with 2,3-butanedione at pH 8.0 in the presence of 50 mM borate in the dark, caused rapid inactivation of sulfate equilibrium exchange. The activity loss followed pseudo-first-order kinetics. This is indicated by the straight line obtained in semi-log

plots of transport rate versus time. The degree of inhibition was dependent on reagent concentration as indicated in Fig. 1. No inhibition takes place when the cells were incubated in the same medium at pH 7.4.

Modification of resealed ghosts with 2,3-butanedione in absence of borate at pH 8.0

Since the role of borate has been reported to be critical for the reaction of butanedione with free arginine or with arginine residues in protein [13], resealed ghosts have been incubated in 10, 20 and 50 mM butanedione in standard medium without borate for 90 min. Table I, shows that under these conditions 10 and 20 mM cause no inhibition. The rate constant for SO_4^{2-} exchange after incubation of the resealed ghosts with 50 mM butanedione for 90 min was $87 \pm 5\%$ of the control value. When these experiments were done in presence of 50 mM borate, the rate constant was $27 \pm 2\%$ of the control value. These results show that within the experimental error, little or no inhibition can take place in absence of borate. This is most probably due to the reversibility of the reaction between butanedione and the arginine residues on the transporter according to the reaction scheme (see Introduction).

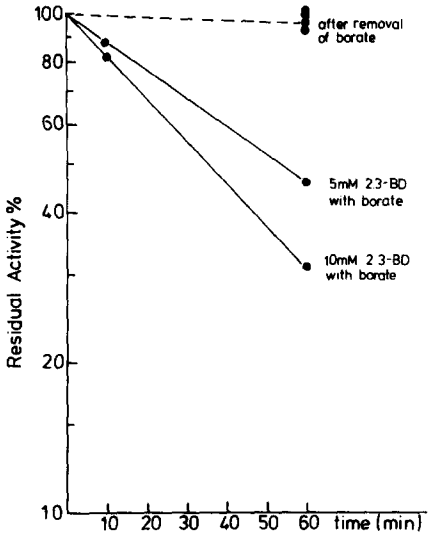


Fig. 1. Semilogarithmic plots of inactivation of sulfate equilibrium exchange by 2.3-butanedione. Resealed ghosts were incubated in standard medium containing 50 mM borate at 37 °C at the concentration of the reagent indicated in the figure. At the times indicated on the abscissa aliquots were withdrawn, excess butanedione was removed by washing, either in presence or in absence of borate and the residual activity of $^{35}SO_4^{2-}$ equilibrium exchange was measured. The ordinate presents the residual flux as % of a control value in the same media used for the reaction but without inhibitor. Flux measurements were done as described in Ref. 1.

TABLE I

EFFECT OF BORATE ON THE RATE OF TRANSPORT INACTIVATION BY 2,3-BUTANEDIONE (BD)

Conditions	Residual activity in % [a]
10 mM BD without borate	100 ± 5
20 mM BD without borate	100 ± 5
50 mM BD without borate	87 ± 5
50 mM BD with borate	27 ± 2

[a] Rate of SO_4^{2-} exchange in % of a control value in the same media without butanedione.

Reversibility of the reaction of 2,3-butanedione with resealed ghosts

After incubation of the resealed ghosts with butanedione in presence of borate as indicated in Fig. 1, the cell suspension was centrifuged to remove excess inhibitor and was washed three times with standard buffer without borate. In some experiments the cells were suspended for 1 h at 37 °C in this buffer before flux measurements. Under both conditions the resealed ghosts recovered all their transport activity after removal of borate.

These results indicate the stabilization of the product between the arginine residues in the transporter and the 2,3-butanedione molecule by borate ions, as indicated in the reaction scheme. The specific enhancement by borate, the reversibility of the modification and the irreversibility in presence of borate clearly demonstrate that the modified residues on the transporter are guanidino groups.

Interaction between the substrate binding site and the binding site of 2,3-butanedione

The ability of chloride ions to protect the transport system against inactivation by butanedione was tested by incubation of the ghosts at different concentrations of cloride ions before adding the modifier under the conditions indicated in Fig. 2.

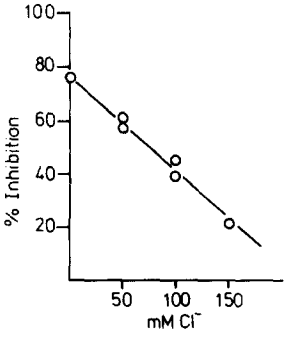


Fig. 2. Effect of chloride ions on the inhibition of $^{35}SO_4^{2-}$ self-exchange by 2,3-butanedione. Resealed ghosts were equilibrated at either 0, 50, 100 and 150 mM Cl^- in standard buffer, containing 50 mM borate and loaded with $^{35}SO_4^{2-}$ prior to exposure to 10 mM 2,3-butanedione at pH 8.0. Inhibition was calculated by comparison with fluxes measured in the same medium without inhibitor.

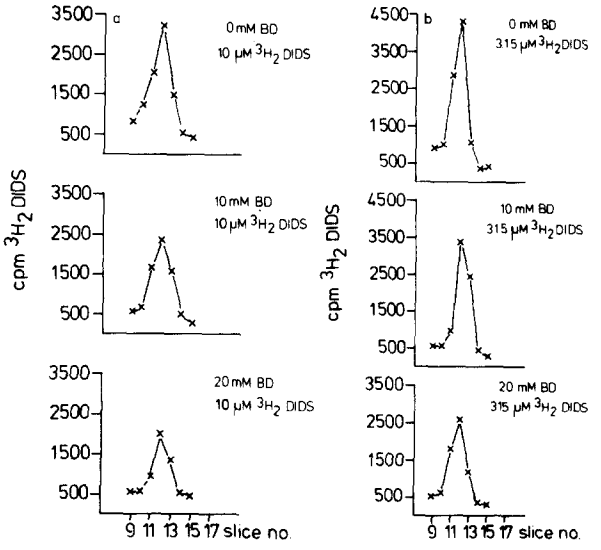


Fig. 3. $^3H_2$DIDS binding pattern to red cell resealed ghosts after modification at either 10 μM $^3H_2$DIDS (Fig. 3a) or 3.15 μM $^3H_2$DIDS (Fig. 3b). Prior to the exposure to $^3H_2$DIDS the ghosts had been treated with 2,3-butanedione. The 2,3-butanedione concentration was 0, 10 mM or 20 mM.

The results show that chloride ions are able to protect the transport system against inactivation with butanedione. The protective effect increases with increasing chloride concentration in the medium.

Interaction between 2,3-butanedione binding site and the binding site of $^2H_2$DIDS

Resealed ghosts were modified at either 10 or 20 mM butanedione for 60 min in borate buffer under the conditions described in Fig. 1. After removal of the unreacted butanedione by washing five times in borate buffer (two washes were containing 0.5% bovine serum albumin) each resealed ghost suspension was subdivided into two portions. One was used for flux measurement, the other for the determination of the capacity of the transporter to bind $^3H_2$DIDS. The incubation with $^3H_2$DIDS was performed at concentration of 3.15, 10 or 20 UgmM for 45 min at 37 °C in the same borate buffer as that used for the butanedione reaction. The membranes of the $^3H_2$DIDS-treated ghosts were isolated, dissolved in SDS and subjected to polyacrylamide gel electrophoresis.

The capacity of band 3 protein for binding of $^3H_2$DIDS was determined as previously described [2]. The results represented in Fig. 3 show that under a $^3H_2$DIDS concentration of 10 μM, the $46 \pm 6\%$ inhibited transporter (10 mM butanedione) can still bind $^3H_2$DIDS up to $85 \pm 4\%$ to its total binding capacity. (In some experiments flux measurements were done after $^3H_2$DIDS treatment and the inhibition of SO_4^{2-} exchange was increased to 99%.)

The $62 \pm 6.4\%$ inhibited transporter (20 mM 2,3-butanedione) can still bind $^3H_2$DIDS to $81 \pm 12\%$ of its binding capacity, Fig. 3a. At 3.15 μM $^3H_2$DIDS, the binding capacity of transporter was $91 \pm 12\%$ after modification with 10 mM 2,3-butanedione, and $79 \pm 14\%$ after modification with 20 mM 2,3-butanedione Fig. 3b. Similar results have been found with 20 μM $^3H_2$DIDS, the binding capacity was $89 \pm 13\%$ after modification with 10 mM 2,3-butanedione and $84 \pm 7\%$ after modification with 20 mM 2,3-butanedione.

These results do not support evidence for steric interaction between the DIDS-binding site and the arginine residue (s) responsible for the inhibition of anion transport after modification with this reagent. As we have mentioned before the reaction between the guanidino group and 2,3-butanedione produces dihydroxyimidazoline which forms a negatively charged complex with borate. Under our experimental conditions (in presence of 50 mM borate), the reaction between

the essential arginine residue(s) and butanedione is stabilized by borate to form the *cis*-diol dihydroxyimidazoline-borate complex and the equilibrium is shifted to the right. One would expect that the relatively bulky and negatively charged complex would have been able to be a hindrance for the binding of $^3H_2$DIDS. $^3H_2$DIDS is known to bind first reversibly to a site which contain at least two positive charged residues on the transporter [15] (electrostatically with its two sulfonic acid groups), then covalently with a lysine group on the 60 kDa fragment of band 3 [16]. Under such conditions a significant reduction of the covalently binding of $^3H_2$DIDS should have been noticed. Our results would suggest that these essential arginine residues may not be located in the immediate vicinity of the $^3H_2$DIDS-binding site.

Effect of 2,3-butanedione on the reversible binding site of $^3H_2$DIDS

Experiments have been done in which resealed ghosts have been modified with either 5, 10, 20 and 50 mM butanedione and in presence of borate buffer. After removal of unreacted modifier by washing, the cells were reincubated with 10 μM $^3H_2$DIDS in a medium containing 50 mM borate for 1 or 5 min at 0 °C. The pH of the medium was 6.5. Under these conditions the interaction of $^3H_2$DIDS with the transporter is largely reversible [18,19]. The $^3H_2$DIDS binding was calculated from the decrease of $^3H_2$DIDS concentration in the medium brought about by the addition of the 2,3-butanedione modified ghosts.

A control was done in which unmodified resealed ghosts have been added to the same $^3H_2$DIDS medium. The results in Table II show that there is no significant reduction in the H_2DIDS binding capacity of the resealed ghosts after modification with 5, 10 and 20 mM butanedione. After modification with 50 mM 2,3-butanedione (inhibition of the transport system was about 75%), a reduction of about 30% has been noticed. These results also provide no evidence for a direct steric interaction between the reversible $^3H_2$DIDS binding site (the two positively charged groups on the transporter which are assumed to be the site of interaction with the two sulfonic acid groups of the H_2DIDS molecule) and the essential arginine (s) residue which react with butanedione. On the other hand the scattering of the data of the number of the covalently binding $^3H_2$DIDS molecules to band 3 after modification with butanedione and the reduction of about 30% of the reversible $^3H_2$DIDS binding at 0 °C after 75% inhibition of the transport system may provide some evidence for an interaction between the two binding sites. These interactions are most probably nonsteric. Alternatively if both inhibitors produce their inhibitory effect by acting on the same site, one must assume that the H_2DIDS molecule must be able to displace the arginine-butanedione complex, which seems to be unlikely under our experimental conditions due to the presence of borate. Now a question arises about the location of these essential arginine. In case that both borate and butanedione are able to penetrate the cells, the inhibitory site can also be located on the inner surface of the membrane. More studies are now being done to characterize these residue(s) in more detail.

TABLE II

THE EFFECT OF THE PRETREATMENT OF THE RESEALED GHOSTS WITH BUTANEDIONE (BD) ON THE REVERSIBLE BINDING OF $^3H_2$DIDS

Conditions	% of the reversibly bound $^3H_2$DIDS
Unmodified resealed ghosts	100%
5 mM BD modified ghosts	92 ± 5%
10 mM BD modified ghosts	80 ± 5%
20 mM BD modified ghosts	76 ± 5%
50 mM BD modified ghosts	71 ± 7%

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