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ANION EXCHANGE IN HUMAN ERYTHROCYTES HAS A LARGE ACTIVATION VOLUME

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Sulfate equilibrium exchange in human red cells has an activation volume of $+150 \pm 20$ cm^3/mol over the pressure range 0.1 to 83 MPa (15 to 12000 lb/in^2) at 30°C. This value greatly exceeds the expected contribution from sulfate binding to the anion exchanger. We suggest that the activation volume reflects conformational changes during the transport cycle.

Introduction

Studies of the effects of high pressure on biological systems are generally useful for one or both of the following: (1) they are used to delineate the problems encountered and the adaptive responses of organisms exposed to hyperbaric environments, and (2) they provide insight into mechanical changes (e.g., changes in volume) associated with biological action at atmospheric pressure. Although the pressure dependence of enzyme-catalyzed reactions has been widely used to probe reaction mechanisms, only a few studies have treated effects of pressure on membrane transport. Hall et al. [1] examined several components of K^+ transport in human red cells, and several studies have examined the pressure dependence of the $(Na^+ + K^+)$-ATPase [2] and Ca^{2+}-ATPase [3]. However, with the possible exception of one qualitative study of the effects of pressure on phosphate exchange [4], there is no information regarding the pressure dependence of anion transport in red cells, despite the fact that the red cell anion transport appears to be a particularly attractive candidate for pressure studies. The anion exchange protein, band 3, is better characterized than any other red cell membrane transport protein; it has an unusually large activation energy and its cyclic activity is presumed to involve conformational change. Estimates of volume changes during its activity should provide critical constraints on any theory of action.

This paper contains results of our studies on the pressure dependence of sulfate exchange in human red cells. In particular, we find the overall activation volume for sulfate exchange to be $+150$ cm^3/mol, which is the largest value reported for any transport system.

Methods

Blood was obtained by venipuncture from paid human donors into a citrate/dextrose/phosphate solution and was used within 1 week of collection. Blood was adjusted to the proper pH with 300 mM HCl and the red cells were separated from the plasma and buffy coat by centrifugation and resuspension three times in unbuffered isotonic saline.

Sulfate fluxes were measured by following $^{35}SO_4$ efflux under conditions of equilibrium exchange in

Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

sulfate-loaded cells. Cells were loaded by repeated suspension in sulfate medium at 35°C, allowing sufficient time for sulfate to exchange with the intracellular chloride. The medium consisted of 95 mM K_2SO_4/5 mM Na_2SO_4/27 mM sucrose/10 mM buffer (either Mops-KOH at pH 6.65 or Tris-H_2SO_4 at pH 7.00). In each flux experiment, 0.2 ml isotope-loaded cell suspension at 0°C was suspended in 10 ml of ice-cold medium and centrifuged once to remove external label and then resuspended in 10 ml medium at 30°C at a final hematocrit of about 1%. At 10–20 min intervals, samples were removed and rapidly centrifuged through a dibutylphthalate cushion; entire samples of the cell suspension were used for the asymptotic points. Aliquots of the supernatant or suspension were deproteinized with trichloroacetic acid and counted by liquid scintillation using Aquasol II (New England Nuclear).

The cell suspension was pressurized in an open acrylic cup enclosed within a gas-free pressure vessel, using tetradecane as the hydraulic fluid. Control experiments showed that tetradecane was without effect on transport. Pressures were generated using a hand-operated pump (Enerpac model 11-400). The system could be pressurized to 83 MPa (12000 lb/in^2) in less than 6 s, and pressure could be reduced to atmospheric in well under 1 s. Samples were removed through a valve connected to a flexible tube extending into the cell suspension. Typically, the chamber was depressurized for 0.5 min during the sampling period.

Estimation of rate constants

Since it is necessary to depressurize the system to withdraw samples, a portion of the observed efflux occurs at atmospheric pressure rather than at the experimental pressure. Because transport decreases with pressure, the rates are overestimated if no correction is applied, and this overestimation increases with pressure. When pressures in the system are changed, there is an immediate temperature change followed by a relaxation as the system equilibrates thermally. These temperature changes prevent a simple correction of the observed rates for the efflux during the depressurized sampling intervals. For that reason, rate constants were estimated by a nonlinear least-squares method which takes into account both the low-pressure sampling interval and the effects of temperature. The data are fitted to the numericallly integrated form of the differential equation

$$\frac{dA}{dt} = -k(T,P)(A - A_\infty) \tag{1}$$

where A is the external tracer activity and A_∞ its asymptotic value. The rate constant $k(T,P)$ is given by

$$k(T,P) = k(T_{ref},P)\exp\left\{-\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)\right\} \tag{2}$$

where E_a (144 kJ/mol) is the measured activation energy for sulfate exchange and T_{ref} is the reference temperature of 30°C. The rate constant at atmospheric pressure and temperature T_{ref} is measured separately. Temperatures in the apparatus were approximated by a simple exponential model

$$T = T_{ref} + (T_n - T_{ref})e^{-\lambda(t-t_n)} \tag{3}$$

where T_n and t_n are the initial temperature and time in the nth constant pressure interval. Each T_n (except the first, T_0) is obtained from the temperature at the end of the previous interval by adding a step temperature change of β or $-\beta$, depending on whether the pressure is being increased or decreased. The parameters T_0, β, and λ were determined in parallel experiments in which temperatures were monitored with a thermistor. Eqns. 1, 2 and 3 were integrated numerically, subject to the assumptions that E_a is independent of pressure and that pressure changes occur instantaneously. The resulting curves are fitted to experimental data by a nonlinear least-squares procedure to determine the best fit value of $k(T_0, P)$ at each pressure. An example of the results of this procedure applied to an experiment at the highest pressure (which requires the largest correction) is shown in Table I; the data are satisfactorily fitted to within 1.5% by a single rate constant.

Results

Anion exchange is strongly decreased at high pressure (Fig. 1). The inhibition observed in these experiments is reversible; cells held at 83 MPa for 1 h yielded rates nearly identical to controls after

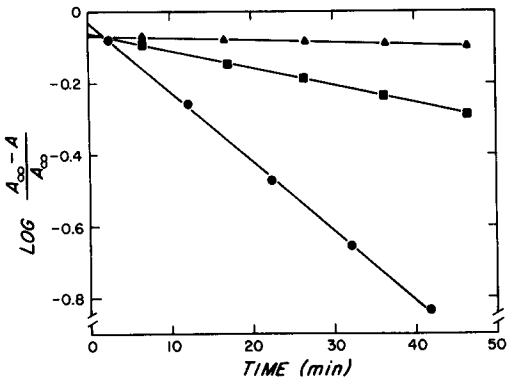

Fig. 1. Time-course of $^{35}SO_4$ efflux at several pressures in Mops buffer. (●) atmospheric pressure, (■) 21 MPa, (▲) 83 MPa. A is activity in the supernatant. Lines do not intersect at origin because the single wash did not completely remove external label and (in the case of experiments at pressure) several minutes elapsed before the initial pressurization.

pressure was reduced to 0.1 MPa (91% ± 2% of control, $N = 3$).

These results are not merely an effect of pressure on pH. The pK for Tris decreases by less than 0.04 unit at the highest pressures used [5,6]; a pH difference of this magnitude should, by itself, cause an increase in rate of about 10% [7]. The pressure dependence of Mops is not known but is believed to be small, as is the case for most amines. Larger variations in internal pH with pressure cannot be ruled out.

The log of the rate constant varies linearly with pressure over the entire range studied (Fig. 2A). The activation volume (ΔV^*)

$$\Delta V^* = -RT\frac{d \ln k}{dP} \quad (4)$$

estimated from the data is $+150 \pm 10$ cm^3/mol in the 0.1–83 MPa range (from slope and standard error of the regression) for both buffers. The actual uncertainty in this value is probably larger, as is discussed below.

The greatest error appears to arise from the corrections applied for efflux during the sampling period. The estimation of rate constants is quite insensitive to the assumption that the E_a is independent of pressure because temperatures were always close to T_{ref}, and it is insensitive to small errors in most of the other factors in the analysis. However, the correction is quite sensitive to the estimate of temperature during the depressurized sampling intervals because a small error in the portion of the flux assigned to the sampling period results in a proportionally larger change in that assigned to the experimental pressure. In the worst case, at the highest pressure used, approx. 80% of the flux occurs at atmospheric pressure (Table I). A temperature error of 1.0°C (a reasonable upper bound for this uncertainty), which results in a 20% error in the calculated efflux at atmospheric pressure, causes an error of factor 2 in the estimated rate constant at 83 MPa. This error is small in comparison to the 140-fold difference in rate between this pressure and atmospheric pressure and will result in an error in the estimated activation

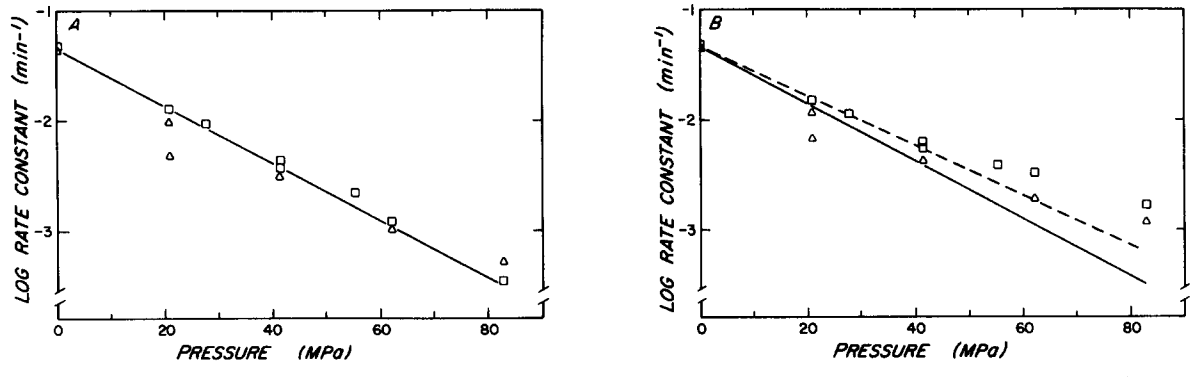

Fig. 2. Dependence of rate constant for sulfate ion equilibrium exchange on hydrostatic pressure. (□) pH 6.65 in Hepes buffer, (△) pH 7.00 in Tris buffer. (A) Corrected rate constant calculated as described in text. The line is the best fit corresponding to $\Delta V^* = 150$ cm^3/mol. (B) Uncorrected rate constant. Solid line as in (A), dashed line corresponds to 130 cm^3/mol.

TABLE I

EXAMPLE OF FIT TO DATA FOR SULFATE FLUX EXPERIMENT AT 83 MPa

The estimated rate constant is $(3.50 \pm 0.64) \cdot 10^{-4}$ min^{-1}. Asymptotic activity is 7193 cpm. The standard error of the fit is 12 cpm. The estimated efflux during the sampling interval is given by the difference between columns B and A.

Time [a] (min)	Calculated activity (cpm)			Observed activity (cpm)	Relative error [e] (%)
	Release [b]	Repressurization [c]	Sample [d]		
	A	B	C	D	
6.7	847	996	1055	1043	+1.1
16.7	1021	1128	1170	1185	−1.3
26.6	1148	1223	1260	1266	−0.4
36.5	1242	1313	1331	1326	+0.4
46.5	1331	–	1418	1414	+0.2

[a] Time at which flux was stopped by centrifugation through dibutylphthalate.
[b] In pressure cell at the at the beginning of the depressurized sampling interval.
[c] In pressure cell at the end of the sampling interval.
[d] Calculated for sample at time of centrifugation.
[e] Column C compared to column D.

volume of about 20 cm^3/mol. Another estimate of the error may be obtained from the rate constants calculated without making any corrections for flux during the sampling intervals. Because the corrections necessarily raise the calculated activation volume, this estimate can be used as a lower bound for $\Delta V^{\neq}$. A value of approx. 130 cm^3/mol satisfactorily fits the data at the lower experimental pressures, where the correction is small (dashed line in Fig. 2B). Thus, a reasonable estimate of the activation volume in these experiments is $+150 \pm 20$ cm^3/mol.

Discussion

The activation volume of 150 cm^3/mol reported here is larger than that observed in any simple chemical reaction and is larger than those for almost all enzyme reactions. For example, in Morild's compilation of about 100 $\Delta V^{\neq}$ values for enzyme reactions [8], the majority are between -30 and $+30$ cm^3/mol, and only one, citrate synthase from the abyssal fish *Antimora*, exceeds 75 cm^3/mol. Most of these are soluble enzymes and may therefore be poor models for transport proteins. Some of the few membrane transport systems studied to date have large activation volumes: $+65$ cm^3/mol for active K^+ transport in human red cells [1]; $+90$ to $+100$ cm^3/mol (for the low-temperature or high-pressure region) for $(Na^+ + K^+)$-ATPase from porcine kidney [2]. However, lower values have been obtained from other $(Na^+ + K^+)$-ATPase preparations [8].

In addition, a large negative $\Delta V^{\neq}$ of -87 cm^3/mol has been observed for the inhibitor-insensitive K^+ leak (into chloride media) in human red cells [1]. This K^+ leakage is presumably a simple process; however, interpretation of its pressure dependence is complicated by the existence of a number of silent but potential leak pathways (e.g., leaks stimulated by mercurials, butanol, and internal Ca^{2+}). This raises the possibility that the pressure-stimulated K^+ leak is not simply an activation of a single process, but may result from the recruitment of additional leakage units.

There is currently no satisfactory general theory to explain large activation volumes in enzyme-catalyzed reactions or protein-mediated transport. One general feature of membrane transport absent from soluble enzymes may be the involvement of the lipid. In the kidney ATPase, changes in the position of a distinct breakpoint in the Arrhenius plots with pressure suggest the involvement of phase transitions in the boundary lipids [2]. There is some evidence of a role for lipid-protein interactions in anion transport: cholesterol enrichment decreases sulfate transport, but cleavage of membrane phosphatidylcholine by phospholipase A_2

has no significant effect [9]. In our experiments, there is no evidence for phase transitions and it is premature to estimate the magnitude of the contribution that lipid-protein interactions make to the observed activation volume.

Several other mechanisms for volume changes associated with transport are possible. In this system, the binding of sulfate to a cationic binding site should contribute a positive component to the $\Delta V^{\neq}$ due to the larger electrostriction of water around the fully hydrated sulfate ion compared to the bound ion. Likewise, binding of the proton that is transported with the sulfate ion could contribute to the observed activation volume. Similarly, binding of sulfate to the inhibitory 'modifier' site and protonation of an inhibitory site may contribute to the observed activation volume but, in this case, a positive volume change for binding would act in the opposite direction and decrease the observed activation volume. The ΔV for proton binding to various groups ranges between -6 and $+23$ cm^3/mol; volume changes for sulfate binding probably do not exceed 20 cm^3/mol (a rough estimate based primarily on values for pair formation between sulfate and inorganic cations; we have found no published values for sulfate binding to proteins or model compounds). Finally, the contribution to the observed activation volume will be less than the volume change for binding alone, depending on the details of the kinetic mechanism. For example, if the rate of transport is proportional to the fractional occupation of the binding site, and if binding is rapid compared with the subsequent translocation steps, then we may describe transport by

$$J = \alpha\left(\frac{[S]}{K_d + [S]}\right) \tag{5}$$

where J is the flux, [S] is the sulfate concentration, K_d is the dissociation constant, and α is a constant of proportionality which may include terms for binding to the modifier site. Differentiating $\ln J$ with respect to pressure P yields

$$\frac{d\ln J}{dP} = -\frac{K_d}{K_d + [S]}\frac{d\ln K_d}{dP} + \frac{d\ln \alpha}{dP} \tag{6}$$

By substituting the expression for activation volume (4) we obtain

$$\Delta V^{\neq} = \frac{K_d}{K_d + [S]}\Delta V_b + RT\frac{d\ln \alpha}{dP} \tag{7}$$

where ΔV_b is the volume change for binding. Using published values for K_d for sulfate of 30 to 40 mM [7,10], the fraction of the volume change for binding that contributes to the overall activation volume, $K_d/(K_d + [S])$, should be about 0.3.

In addition, formation of salt bridges such as those postulated in the model of Wieth et al. [11] could contribute a volume change on the order of 30 cm^3/mol [12]. It would be incorrect, however, to attempt to add up such volume changes for each step, except in the energetically unlikely case that all the salt bridges were formed or broken simultaneously.

By themselves, these ionic volume changes are too small to account for the observed $\Delta V^{\neq}$. Several studies of subunit interactions in multi-subunit enzymes have obtained large ΔV values [12]. Although there is substantial evidence for the existence of dimers of band 3, the protein responsible for anion transport in red cells, there is no evidence for a functional role for the dimers in transport. No cooperation is seen in halide or sulfate transport and estimates of binding sites using labelled inhibitors appear to indicate one site/monomer [13]. Further, all of the observed large ΔV values associated with subunit interactions occur in soluble enzymes, in which dehydration of protein surfaces during polymerization is probably a major contributor to the volume changes. Dimerization within the membrane is less likely to exhibit such effects.

Reversible denaturation of band 3 by pressure is also unlikely to explain the observed $\Delta V^{\neq}$ for sulfate exchange. Although pressure-induced structural changes in proteins may have large ΔV values, typically, pressures of 200 to 300 MPa are required before significant changes are observed [14].

The most likely explanation for large volume changes during membrane transport is that ion binding and translocation require conformational changes and that the conformational changes involve large volume changes. The observed ping-pong kinetics for anion exchange seem to require

two distinct stable conformations for the exchange protein; one in which the binding site faces the intracellular compartment and one in which it faces the extracellular compartment. Indirect evidence for conformational changes has come from studies of changes in reactivity of several groups on band 3, which have been interpreted as changes in exposure [15]. It is often assumed that only a small portion of the protein molecule participates in the conformational change; the large activation volume observed here suggests a more extensive rearrangement.

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References

1 Hall, A.C., Ellory, J.C. and Klein, R.A. (1982) J. Memb. Biol. 68, 47–56
2 De Smedt, H., Borghgraef, R., Ceuterick, F. and Heremans, K. (1979) Biochim. Biophys. Acta 556, 479–489
3 Heremans, K. and Wuytack, F. (1980) FEBS Lett. 117, 161–163
4 Murphy, R.B. and Libby, W.F. (1976) Proc. Natl. Acad. Sci. USA 73, 2767–2769
5 Distèche, A. (1972) Symp. Soc. Exp. Biol. 26, 27–60
6 Neuman, R.C., Jr., Kauzman, W. and Zipp, A. (1973) J. Phys. Chem. 77, 2687–2691
7 Schnell, K.F., Gerhardt, S. and Schöppe-Fredenburg, A. (1977) J. Memb. Biol. 30, 319–350
8 Morild, E. (1981) in Advances in Protein Chemistry (Anfinsen, C.B., Edsall, J.T. and Richards, F.M., eds.), pp. 93–166, Academic Press, New York
9 Deuticke, B., Grunze, M. and Haest, C.W.M. (1980) in Membrane Transport in Erythrocytes, Alfred Benzon Symposium 14 (Lassen, U.V., Ussing, H.H. and Wieth, J.O., eds.), pp. 143–156, Munskgaard, Copenhagen
10 Barzilay, M. and Cabantchik, Z.I. (1979) Membrane Biochem. 2, 255–281
11 Wieth, J.O., Bjerrum, P.J., Brahm, J. and Andersen, O.S. (1982) Tokai J. Exp. Clin. Med. (Suppl.) 7, 91–101
12 Heremans, K. (1982) Annu. Rev. Biophys. Bioeng. 11, 1–21
13 Knauf, P.A. (1979) Curr. Top. Membranes Transp. 12, 249–363
14 Weber, G. and Drickamer, H.G. (1983) Q. Rev. Biophys. 16, 89–112
15 Passow, H., Kampmann, L., Fasold, H., Jennings, M. and Lepke, S. (1980) in Membrane Transport in Erythrocytes, Alfred Benzon Symposium 14 (Lassen, U.V., Ussing, H.H., and Wieth, J.O., eds.), pp. 345–367, Munskgaard, Copenhagen