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Temperature dependence of the exchange of monovalent anions in human red blood cells

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Abstract

The temperature dependence of anion exchange across the red cell membrane was studied between 5°C and 55°C by measuring the rate of shrinkage of cells when transferred from a medium of pH 7.6 to one of pH 9.3 (as measured at 22°C). The rates of shrinkage varied with the anion studied, the order being $F^- > Cl^- > Br^- > I^- > SCN^-$ but were faster in the presence of trace amounts of carbon dioxide than in its absence. NO_3^- was as fast as Cl^- when carbon dioxide was present but comparable with I^- when it was removed. Arrhenius plots of the rates were linear for all anions over the temperature range studied and gave the following apparent activation energies in kJ · mol^{-1}: F^-, 67.7; NO_3^-, 68.4; Cl^-, 70.2; Br^-, 79.6; SCN^-, 87.4 and I^-, 95.1 in the presence of carbon dioxide. Inhibition of carbonic anhydrase with 5 μm ethoxzolamide and the removal of the carbon dioxide by degassing raised the activation energies to: F^-, 124.8; NO_3^-, 129.0; Cl^-, 141.5; Br^-, 159.4; SCN^-, 150.0 and I^-, 185.6 kJ · mol^{-1}. With the exception of F^-, the apparent activation energies of the anions were linearly related to their thermochemical (dehydrated) radii in both cases. The relationship between the ionic radii and the energy of transfer suggests that anion exchange involves transfer through a hydrophobic pathway and that additional energy is required to overcome restrictions experienced in passing through the pathway. It is proposed that this, rather than a conformational change in the protein determines the activation energy of the process.

Keywords: Red cell; Anion exchange; Activation energy

1. Introduction

The mechanism of the band 3 anion exchanger in the red cell membrane is of interest since the turnover rates of the transporter are very high, of the order of $4 \cdot 10^3$ ions/band $3 \cdot s^{-1}$ at 15°C [1]. Kinetic studies show that the exchanger operates by a ping-pong mechanism, that is, the anions are translocated alternately from one side of the membrane and then from the other in a one-for-one exchange [2]. Such behaviour is conventionally explained in terms of an alternating conformer model in which a change in the conformation of the protein moves the anion across the membrane (reviewed in Ref. [3]). Alternating conformations are found in other transport systems, notably the glucose transport system of human red cells [4]. Studies of the mechanism of this system by Widdas and Baker [5] showed that the change in conformation derived its energy from the surface energy of water. The movement of the protein domains was resisted by the internal friction of the molecule and this was the main factor responsible for the relatively slow rate of turnover, about 50 s^{-1}, [6] or almost a hundred times less than that of the anion exchanger at the same temperature. Thus the confor-

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mational changes in the glucose transporter cannot occur fast enough for anion exchange and it seems improbable that the same mechanism could be operating in both types of transporter.

As an alternative, and bearing in mind the special properties of arginine and glutamic acid residues, both of which have been implicated in the anion exchange process [7–9], Widdas and Baker [10,11] proposed a mechanism for the anion exchanger built around an electrophoretic oscillator. This was envisaged as an hydrophobic channel containing a linear chain of *N*-arginine guanidinium groups stabilised by $N-1$ semi-fixed anions (carbonyl groups), where N is an odd number. Since the guanido groups carry a positive charge at physiological pH ($pK = 12.4$) there is one unmatched proton in the chain. Switching of this unmatched proton from one guanido nitrogen to the next draws an anion along the chain. The movement of the proton back and forth from one end of the chain to the other corresponds to the conformational change and because there is only one unmatched proton, only 1:1 exchanges of anions are possible. At either end of the electrophoretic channel is a target zone that strips the anions from the water interface and holds them ready to enter the oscillator. Beyond the water interface is the cleft that is rich in lysines and excludes cations.

In such a model it might be expected that the translocation process would depend on the physical properties of the anion. Differences in the rate of transfer of different anions were noted by Gunn [12] but could have been due to differences in uptake by the exchanger. Since the activation energy of transfer depends on the rate-limiting step in the process and Falke et al. [13] have shown by NMR analysis that the translocation step is rate-limiting in anion exchange, the determination of the activation energies of a range of anions could provide further insight into the working of the band 3 protein.

Such a study of activation energies has already been made by Dalmark and Wieth [14] using radioisotopes. They obtained values for the apparent activation energies of between 120 and 160 kJ · mol^{-1} for several monovalent anions but while bromide and iodide had higher activation energies than chloride and thiocyanate there was no clear relationship with any anionic parameters and the authors did not attach any significance to the differences observed.

Although an exchange process, anion transfer may produce a volume change if the influxing anion is bufferable [15]. In this case the ion (bicarbonate or hydroxyl) can be buffered inside the cell and rendered osmotically inert so that there is a net loss of permanent anion from the cell and a decrease in cell volume. As we have shown elsewhere, this technique can follow shrinkage rates over more than three orders of magnitude and gives highly reproducible results [16]. In this study we have examined the effect of temperature on the rates of exchange of several monovalent anions to try to obtain more information on the translocation process.

A preliminary presentation of some of this work was made to the XXXIInd Congress of the IUPS [17].

2. Materials and methods

2.1. Media

All media were based on the buffered saline normally used in this laboratory. They contained 120 mM NaX, 5 mM KX and 20 mM borate buffer at pH 9.3 (pH measured at room temperature), where X was the anion. pH 7.6 buffer was made by titrating the high pH buffer with phosphoric acid. The anions used, were fluoride, chloride, bromide, iodide, nitrate and thiocyanate. Nitrite and cyanate were also examined but proved unsatisfactory, nitrite because it reacted with the haemoglobin and cyanate because it decomposed producing CO_2.

For the experiments where CO_2 was excluded, the media contained 5 μM ethoxzolamide (Sigma, Poole, Dorset, UK) and were degassed under vacuum for a minimum of 1 h. The degassed media were stored in flasks vented through a container of soda lime until used. Cell suspensions contained a similar concentration of ethoxzolamide and were pre-incubated under reduced pressure for 10 min before starting the experiments.

2.2. Cell preparation

Blood from normal healthy donors was drawn into heparinised tubes and used immediately or on the following day. Before use it was washed with phosphate buffered saline and the plasma and buffy coat

removed. The cells were divided into aliquots, one for each anion, and washed once at high pH in a medium of anion (X) to shrink them and remove endogenous chloride and bicarbonate. They were then washed again, using the anion medium at physiological pH. With the slower moving anions, SCN^- and I^-, the cells were left for 5 min between washes, otherwise nonlinear records were obtained. However even with this precaution, OH^-:I^- exchanges gave a peculiar triphasic response, reminiscent of that seen when cells are put into low ionic strength media [18]. This was avoided if the cells were incubated overnight in iodide media containing 5 μM ethoxzolamide. Measurements of the effect of the polyvalent buffer anions (phosphate and borate) on rate of shrinkage showed that at the concentration used their presence could be ignored [16].

A final suspension of 0.2 ml of the washed cells was made in 10ml of the anion medium at pH 7.6. Although the pH of the incubating medium was some way off from the pK of the buffer, the volume of cells injected was so small that there was no measurable change in the pH of the suspension during use. The suspensions of cells were kept in a water bath at the same temperature as that of the cuvette where the shrinkage was measured, except at the highest temperatures (> 40°C) when they were removed from direct contact with the water.

2.3. Experimental procedure

21 ml of high pH anion medium was placed in the cuvette of a photometer [19], the chart recorder started and 0.15 ml of a suspension of cells loaded with the same anion at pH 7.6 injected into the cuvette. The shrinkage of the cells was followed until complete, the temperature and pH of the cuvette contents were measured with an Orion 811 pH meter, and the procedure repeated. In each experimental session the shrinkage rates for several anions were found over a temperature range from near 5°C to over 40°C in the presence of CO_2 and between 15°C and 55°C in its absence.

2.4. Measurements

After an initial delay of a few seconds for mixing, the shrinkage record is linear until near the final volume. Widdas and Baker [15] showed that the magnitude of the volume change is constant when the pH of the medium lies between pH 9.0 and 11.0 so that the slope of the record is a measure of the rate of loss of a constant amount of anion from the cell. To determine the rate, a line was drawn through the straight part of the record and its slope estimated in chart divisions per min. Calibrations against glucose exits indicated that one chart division was equivalent to approx. 1 mmol of anion per litre of packed cells [15]. To estimate the apparent activation energies, the data for each anion were combined by normalising the lines to the mean logarithmic value at 25°C. Linear regression was carried out on the pooled data for each anion using the Minitab statistical package.

3. Results

3.1. Principle of the technique

In this technique we are measuring an influx of bufferable anions (hydroxyl or bicarbonate) in exchange for an efflux of non-labile or permanent anions [15,20]. These exchanges account for only a small fraction of the total turnover of the transporter (about 0.1%), most of which is permanent anion self-exchange and invisible by the technique since it does not lead to a volume change. The shrinkage rate depends on the relative concentration of the bufferable anion in the external medium as this will decide how large a fraction of the total these exchanges represent. For hydroxyl ions the concentration remains essentially constant over the whole temperature range studied but with bicarbonate ions there is an increase in concentration as the temperature falls. It is this effect that alters the slope of the Arrhenius plot giving a lower activation energy for the transfer process. There are, therefore, two sets of activation energies, a high one due to hydroxyl:anion exchange and a low one determined by bicarbonate:anion exchange [16].

3.2. Temperature dependence of hydroxyl:anion exchange

For this study the experiments were carried out in media that had a pH of 9.3 at room temperature

(22–24°C) as this gave large excursions and reproducible shrinkage rates. Although conditions are far from physiological, it has been shown that the volume changes are linearly related to the pH across the physiological range [15] and estimates of activation energy are similar to those obtained at near-physiological pH [16]. Because of the low concentration of hydroxyl ions, the rates of shrinkage were slow and it was necessary to work at high temperatures, most of the experiments being done done between 15° and 55°C. Six experiments were done with each anion and although there was some variation in the exchange rate with different cell samples, the slopes of the Arrhenius plots for any one anion were similar. Fig. 1 shows Arrhenius plots for one experiment with each anion and it can be seen that they are linear across the temperature range studied. The apparent activation energy for chloride has a value of 141.5 ± 1.7 kJ · mol^{-1}, comparable with the value of 139 kJ.mole^{-1} found by Dalmark and Wieth [14] for isotopic chloride:chloride exchange but much higher than the value of 81.2 kJ · mol^{-1} reported by Lambert and Lowe [21] for bicarbonate:chloride exchange.

Fig. 1 also shows that the slopes of the Arrhenius plots, and thus the apparent activation energies, are different for the various anions. The values range from 124.8 kJ · mol^{-1} for fluoride to 185.6 kJ · mol^{-1} for iodide and are listed in Table 1. Their sequence is similar, for the ions studied, to that found by Dalmark and Wieth [14].

3.3. Rates of hydroxyl:anion exchange

As mentioned above hydroxyl:anion exchange rates are slow, the rate for chloride is about 0.015% of that determined by Brahm [1] for isotopic chloride:chloride exchange. The rates for the various anions differ with fluoride being over twelve times faster at 25°C than thiocyanate (Table 1).

3.4. The temperature dependence of bicarbonate:anion exchange

Fig. 2 shows Arrhenius plots for experiments with each anion in the presence of bicarbonate, measured from about 5°C to over 40°C. Again the plots are linear across the whole temperature range but the

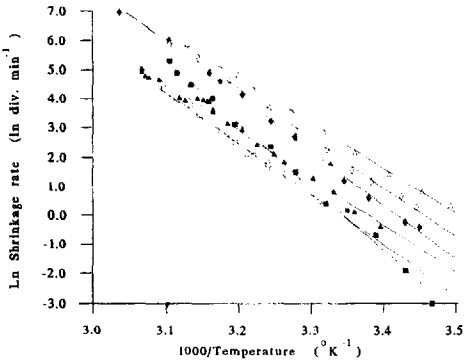

Fig. 1. The temperature dependence of shrinkage rates in the absence of carbon dioxide. Data from one experiment with each anion are shown as an Arrhenius plot of the natural logarithm of the shrinkage rate in chart divisions per min vs the reciprocal of the absolute temperature. The cells and the medium contained the anion at a concentration of 125 mM. △, F^-; ▲, NO_3^-; ◇, Cl^-; ◆, Br^-; □, SCN^-; ■, I^-. The results are given in Table 1.

Table 1
The temperature dependence of hydroxyl:anion exchange for a range of monovalent anions

Anion	Arrhenius plots			Mean rate at 25°C (div · min^{-1})	Apparentactivation energy (kJ · mol^{-1})
	constant (ln div · min^{-1})	slope (ln div · min^{-1} · °K)	r^2		
F^-	52.77 ± 0.83	−15008 ± 252	0.982	11.7 ± 1.6	124.8 ± 2.1
NO_3^-	52.70 ± 0.73	−15513 ± 227	0.965	1.6 ± 0.2	129.0 ± 1.9
Cl^-	58.60 ± 0.67	−17013 ± 205	0.971	4.8 ± 1.4	141.5 ± 1.7
Br^-	65.37 ± 1.35	−19170 ± 415	0.955	3.3 ± 0.7	159.4 ± 3.5
SCN^-	60.34 ± 0.81	−18034 ± 249	0.985	0.8 ± .0.2	150.0 ± 2.1
I^-	74.80 ± 1.05	−22321 ± 327	0.986	1.7 ± .0.8	185.6 ± 2.7

Values were calculated from the linear regression of the pooled data from six experiments for each anion normalised to the mean rate at 25°C. Apparent activation energies were determined from the slope of the Arrhenius plots. 1 div · min^{-1} is approx. equal to 1 mmol of anions · litre of cell water^{-1} · min^{-1} · r^2 is the coefficient of determination.

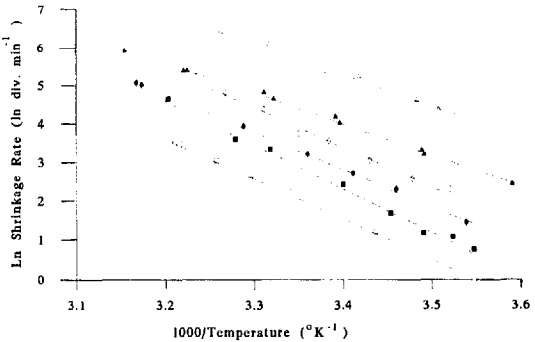

Fig. 2. The temperature dependence of shrinkage rates in the presence of carbon dioxide. Data plotted as in Fig. 1. △, F^-; ▲, NO_3^-; ◇, Cl^-; ◆, Br^-; □, SCN^-; ■, I^-. The results are given in Table 2.

Table 2
The temperature dependence of bicarbonate:anion exchange for a range of monovalent anions

Anion	Arrhenius plots			Mean rate at 25°C (div · min^{-1})	Apparent activation energy (kJ · mol^{-1})
	constant (ln div · min^{-1})	slope (ln div · min^{-1} · °K)	r^2		
F	31.85 ± 0.88	− 8145.4 ± 260.3	0.958	91.5 ± 3.5	67.7 ± 2.2
NO_3^-	31.86 ± 0.65	− 8217.8 ± 191.3	0.972	84.1 ± 2.5	68.3 ± 1.6
Cl^-	32.28 ± 0.85	− 8442.2 ± 251.5	0.962	76.8 ± 3.3	70.2 ± 2.1
Br^-	35.64 ± 0.78	− 9578.2 ± 230.9	0.976	36.9 ± 3.4	79.6 ± 1.9
SCN^-	37.24 ± 0.64	−10512.0 ± 191.3	0.987	7.5 ± 2.5	87.4 ± 1.6
I^-	41.38 ± 0.87	−11442.3 ± 256.4	0.979	21.2 ± 3.4	95.1 ± 2.1

Values, apparent activation energies and rates were calculated as for Table 1.

values of the apparent activation energies which are shown in Table 2, are approximately half of those given by hydroxyl exchange.

3.5. Rates of bicarbonate:anion exchange

The rates of shrinkage in the presence of bicarbonate are about ten times faster than in its absence but the sequence remains the same with the exception of the value for nitrate which is now faster than chloride. Although this disagrees with findings of Dalmark and Wieth [14] and the earlier results of Widdas and Baker [15], the value corresponds to that expected from its activation energy. The differing shrinkage rates with the various anions show that, with the possible exception of fluoride, exchange is not limited by the rate of production of bicarbonate ions.

4. Discussion

4.1. Temperature dependence of anion exchange

Various studies of the temperature dependence of anion exchange in human red cells have given two sets of values for the activation energy of the process [1,2,14,21–24]. We have shown that the lower value of around 70 kJ · mol^{-1} for chloride exchange occurs in the presence of carbon dioxide and is most likely due to changing amounts of bicarbonate in the media altering the observed rate of exchange [16]. In this situation the activation energy depends on the energy of the formation of bicarbonate from carbon dioxide rather than a limiting rate constant in the exchange process [24]. On the other hand, the high value of about 140 kJ · mol^{-1} determined at almost constant hydroxyl concentration is a truer measure of the activation energy for the translocation process. Thus, we believe that the variation in apparent activation energies seen in our hydroxyl:anion experiments reflects real differences in the translocation of the fixed anions. Nevertheless, the results in Tables 1 and 2 clearly show a similar pattern for both sets of apparent activation energies.

4.2. Effect of anion radius

In the current work the obvious differences in the activation energies for the various anions suggest that translocation depends on the properties of the anions. For the halides, there is an increase in apparent

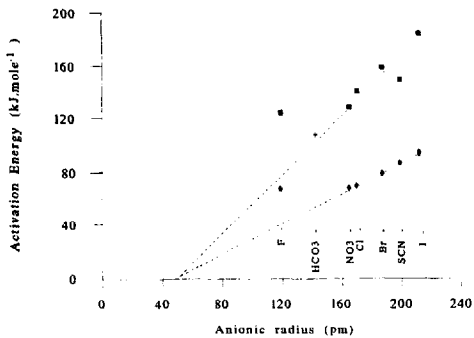

Fig. 3. A graph of the apparent activation energies of anion exchange plotted against the thermochemical (dehydrated) radius of the anion. Bicarbonate:anion results, indicated by ♦ and hydroxyl:anion results by ■, are the mean and S.D. of six determinations. The value of Wieth [23] for radioactive bicarbonate exchange is shown by +. For further discussion see the text.

activation energy with increasing molecular weight but nitrate and thiocyanate do not fit this relationship since their molecular masses are similar, 62 and 58 Daltons, respectively, yet their activation energies are very different.

A better relationship for all the anions is shown when the apparent activation energies are plotted against the thermochemical ionic radii [25] as in Fig. 3. Here the larger ions fall on a single line with a positive slope while the two smallest ions lie on an almost horizontal line. The one exception is the result for thiocyanate:hydroxyl exchange which has a lower activation energy than expected.

The linear relationship of the bicarbonate results means that the activation energy for the exchange of pairs of different anions is determined by that of the larger one and it is the translocation of the larger anion that is the rate-limiting step in the turnover cycle. If the concentrations of bicarbonate in the various media are the same at a given temperature, the apparent activation energies of the anions will be in proportion to their true activation energies. It is only in the case of fluoride that this does not hold because here the larger anion is bicarbonate.

The levelling of the line for the smallest anions suggests that there may be a lower limiting value for the activation energy However, the value of 118 kJ/mole found by Wieth [23] for bicarbonate:bicarbonate exchange falls close to our line for the hydroxyl exchange but is less than that for fluoride, indicating that the transfer of fluoride is anomalous. The thermochemical radii are calculated from crystallographic measurements and are those of the dehydrated ions. The linear relationship suggests that ions must be removed from the water phase for transfer. Of the anions studied, fluoride has the highest hydration energy [26] due to its tendency to organise the water structure around it and, therefore, it may be the dehydration step that is rate-limiting for fluoride exchanges.

4.3. Transfer rates

Contrary to Dalmark and Wieth's [14] assertion that there was no relationship between transfer rate and activation energy, we find that the rates of exchange are related to ionic size and therefore, also to the activation energies. Fig. 4 shows the logarithm of the shrinkage rate for each anion plotted against the anionic radius. Except for those of thiocyanate and nitrate, the points are fitted by a straight line.

Thiocyanate has a much lower exchange rate than

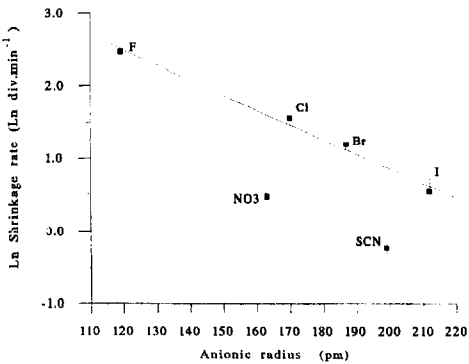

Fig. 4. A plot of the ln rate of hydroxyl:anion exchange at 25°C versus the anionic radius. The points are the means and S.D. of six determinations.

expected for its size and this may be due to its shape. Unlike all of the other anions that are spherical or nearly so, thiocyanate is a linear molecule. Thus we can envisage that a thiocyanate molecule approaching the exchange pathway will only enter if its long axis is appropriately aligned. As it may approach in any one of three orientations, only one third of the molecules will enter the pathway and the rate will only be one third of that expected. The case of nitrate is more puzzling. Hydroxyl:nitrate exchange is very much slower than that predicted from its activation energy and it is difficult to see why, since nitrate gives the expected rate when bicarbonate is present. The fact that the activation energy has the same value relative to the other anions, whether bicarbonate is present or not, suggests that, as with thiocyanate, the effect is due to a reduction in the amount of anion crossing the membrane rather than to a difference in its mode of translocation. One factor that may be relevant is that both nitrate and bicarbonate ions are the same shape. Thus it may be that nitrate is somehow trapped or bound in the cleft of the exchanger but can be displaced by bicarbonate.

4.4. Properties of the exchange process

The dependence of anion exchange on the size of the transported anion has important implications for models of the transporter. The conventional view of the process is that the protein operates by a ping-pong mechanism and that it has two forms, one inward-facing and one outward-facing. Anions are bound alternatively on one side of the membrane then on the other as the protein changes conformation and translocates them between the cell water and the extracellular medium [2,27] In this scheme it is assumed that the translocation of the bound anion through the membrane is the rate-limiting step and the binding of anions at each interface occurs rapidly and reversibly. NMR studies have confirmed that the translocation of the anion is indeed the rate-limiting step [13] and that diffusion to, and association with, the translocation mechanism is much faster. Glibowicka et al. [24], also using NMR, have confirmed that the degree of uptake is unaffected by temperature.

The results of the present study show that for all the anions but fluoride, the energy of transfer is linearly related to the size of the dehydrated ion and therefore, the translocation of the anion occurs by a process that is size-dependent. While this is not incompatible with the operation of a conventional conformational change it does suggest that such a change is unlikely to involve the movements of large sections of protein since these would dominate the mechanics of the process. Furthermore, the dependence of the apparent activation energy on the dehydrated radii shows that the exchange pathway must be hydrophobic, not water-filled. This is consistent with the observation of Cabantchik et al.[28] that there is a hydrophilic-hydrophobic interface within the transport domain of the exchanger and precludes the possibility that the exchanger works in the same way as the glucose transporter [5]. The problem is, therefore, how the exchanger obtains the energy for its operation.

4.5. Thermodynamics of anion exchange

The special characteristic of the band 3 exchange is its very high rate of transfer. Brahm [1] has esti-

Table 3
Calculated values for the half-cycle times and activation entropies of the halide anions at 25°C using the hydroxyl exchange data

Anion	OH:anion full cycle time ($s \cdot div^{-1}$)	Anion half-cycle time ($s \cdot div^{-1}$)	Ln rate anion transfer ($\ln div \cdot s^{-1}$)	ΔS ($J \cdot °K^{-1} \cdot mol^{-1} \cdot$ band 3^{-1})
F^- (OH^-)	5.18	2.6	−0.956	+204
Cl^-	12.5	9.9	−2.293	+250
Br^-	18.2	15.6	−2.747	+306
I^-	35.3	32.7	−3.487	+388

The half-cycle times are the reciprocals of the rates minus half of the reciprocal of the hydroxyl:fluoride rate. Hydroxyl concentration was $2.5 \cdot 10^{-5}$ M. Band 3 concentration was calculated as $1.83 \cdot 10^{-5}$ M from $1.11 \cdot 10^{13}$ cells $\cdot l^{-1}$ and 10^6 transporters $\cdot$ cell^{-1}. The entropy is given as per band 3 transporter to allow comparison with [24].

mated the turnover number for chloride exchange as $3.7 \cdot 10^3$ ions $\cdot s^{-1}$ at 15°C. Since the true activation energy for this process is 141.5 kJ $\cdot$ mol^{-1}, the rate at 25° is about $2.7 \cdot 10^4$ ions $\cdot s^{-1}$ and from our results (see Table 3) we calculate that fluoride is transferred nearly four times as fast as chloride or $1.0 \cdot 10^5$ ions $\cdot s^{-1}$ at this temperature. Thus each full cycle lasts about 10 μs and each translocation must therefore last less than 5 μs. This is very rapid indeed, and again supports the idea that any conformational change is limited in extent since this is the time scale associated with the movement of disordered polypeptide chains [29]. As the complexity of the protein increases so does the internal friction, slowing the movement so that rates of conformational interconversion fall to 10^2 to 10^4 per s [30].

The association of high rates of transfer with high activation energies raises a problem since the fraction of ions in the medium with sufficient energy to be translocated will be given by the term, $\epsilon^{-2\Delta E/kT}$, where ΔE is the activation energy, k is Boltzmann's constant and T is the absolute temperature. Thus the probability of transfer decreases as the exponent of the energy.

In their analysis of the thermodynamics of anion exchange Glibowicka et al. [24] used the transition state theory of absolute reaction rates to interpret their data. The rate of transport, r is given by:

$$r = \kappa \frac{kT}{h} \epsilon^{\Delta S^{\ddagger}/R} \epsilon^{\Delta H^{\ddagger}/RT} [A][C]$$

where κ is the transfer factor, assumed to be 1, k and T, are as defined above, h is Planck's constant, R, the gas constant, $\Delta S^{\ddagger}$, the entropy of the activated state and $\Delta H^{\ddagger}$, its enthalpy (equal to $E_A - RT$). [A] and [C] are the molar concentrations of the anion and transporter, respectively.

It can be seen from this equation that the high rate of transfer and high activation energy can be accomodated by a high positive entropy and Glibowicka et al. [24] suggest that the conformation change is associated with the loosening of bonds both within the protein and in the surrounding medium. We can calculate the transition state entropy for the four halides if we assume hydroxyl ions are translocated at the same rate as fluoride ions (since they are the same size). This allows us to estimate the individual rates of anion transfer and we can then substitute the values in the above equation. The calculation gives the values in Table 3. We can see that like the enthalpies, the entropies also increase with ionic radius. Again it is difficult to understand why small

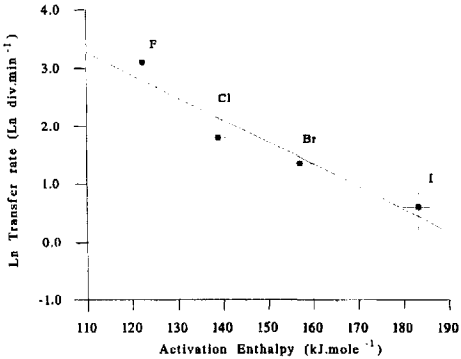

Fig. 5. A graph of the ln rate for anion transfer at 25°C for the four halides plotted against their respective activation enthalpies, ($E_A - RT$). The transfer rates were worked out as in Table 3 and the points lie on a line which has a slope of 0.0387 kJ^{-1} or 1/10 RT.

anions should cause such marked changes in the energetics of a large protein and one is forced to consider the validity of this approach.

An alternative explanation for the high activation energies, suggested by Widdas [31], is that translocation is not a single event but consists of a series of events of low energy each raising the energy of the anion to a higher value. This is similar in some ways to the concept of conformational substates used to determine protein dynamics [32]. The frequency of the events would be high but so would the overall activation energy. Support for this suggestion is seen in Fig. 5 where the shrinkage rates of the halides are plotted against their activation energies. Activated rate theory suggests that a plot of ln rate against activation energy should have a slope of $1/RT$. The line in Fig. 5 has a slope of 0.039 kJ^{-1}, or $1/10$ RT so that the rate appears to depend on only one tenth of the apparent activation energy.

4.6. Interpretation of the results

The results of this study are consistent with the model proposed by Widdas and Baker [10,11]. The linear relationship of the activation energies to the dehydrated radii of the anions supports the view that the transfer of the anion takes place through an hydrophobic pathway and that this is of limited dimensions. A larger anion would encounter more friction within the pathway and would need more energy to pass through it. The logarithmic relationship between the apparent activation energies and the transfer rates confirms that the exchanger uses thermal energy but the slope of less that $1/RT$ suggests that translocation is not a single event but a series of additive steps. This again is in line with the model in which transfer involves the sequential switching of a proton along a row of arginine residues, each switch accelerating the anion.

There remains one piece of evidence that is often quoted in support of a large displacement during the conformational change [24,33], namely the value of the activation volume. An estimate of the activation volume of the exchanger by Canfield and Macey [34] produced a value of 150 $ml \cdot mol^{-1}$, which is higher than that for any other transporter system. However, the activation volume is a measure of the pressure sensitivity of the transfer process. An hydrophobic channel in a protein would tend to collapse under pressure since there is no water to transfer an equalising pressure to the inside. Because, as we have shown, the transfer rate is sensitive to anion size compressing the channel would have the same effect as increasing the anion radius and would slow the transport rate. Thus this result also can be explained by the model.

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