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Inhibition of glucose transport in human erythrocytes by ubiquinone Q_0

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Searches of the protein data bases revealed limited homologies between several regions of the human erythrocyte glucose transporter containing a relative abundance of hydrogen-bonding amino-acid side chains, and proteins of the NADH–ubiquinone oxidoreductase family. This raised the possibility the binding sites for glucose and ubiquinone may be similar in the respective proteins. Experimental studies demonstrated that ubiquinone Q_0 does in fact inhibit both glucose entry and glucose exit in human erythrocytes with kinetics consistent with the existence of ubiquinone binding sites at both the exofacial and endofacial sides of the transporter. Glucose transport was also inhibited by the water-soluble tryptophan-inactivating agent, dimethyl(2-hydroxy-5-nitrobenzyl)sulphonium bromide, and this is consistent with the presence of tryptophan residues in two of the exofacial amino-acid sequences proposed as candidates for involvement in glucose binding sites.

Introduction

Glucose transport in human erythrocytes is among the most extensively studied facilitated diffusion systems. The membrane protein responsible for this transport has been sequenced [1] and belongs to a large family of closely related sugar transport proteins [2,3]. The pattern of hydrophobicity in the amino acid sequence suggests that there may be 12 transmembrane α-helices, a large extramembranous endofacial domain, and a smaller (glycosylated) exofacial domain. These domains are indicated in Fig. 1.

In this report we describe amino-acid sequence homology studies which led us to identify ubiquinones as potential new inhibitors of glucose transport, and transport studies which show that ubiquinone Q_0 inhibits both glucose influx and efflux in human erythrocytes with kinetics which are consistent with the existence of two distinct binding sites for glucose at the endo- and exofacial surfaces of the transporter.

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Methods

Amino-acid sequence homology scans were performed using the OWL suite of programs running on the SEQNET facility at the SERC, Daresbury, U.K. OWL itself is a non-redundant protein sequence data base prepared from the NDRF-PIR, SWISSPROT, GENBANK, NEW, NEWAT68, PSD-KYOTO and BROOKHAVEN data bases [4]. The protein alignments and homology searches were performed using the program, SWEEP, which searches the OWL database for entries which exhibit homology with any selected test sequence. SWEEP is an extension of the NBRF-OPIR FASTP program which uses the Lipman-Pearson algorithm [5].

Human erythrocytes were prepared, and the zero-trans entry of [^{14}C]-D-glucose into glucose-free cells was measured as described by Lowe and Walmsley [6]. Zero-trans exit of glucose was measured by a method similar to that used for entry except that cells were preloaded with [^{14}C]-D-glucose and a selected concentration of unlabelled D-glucose, then concentrated by centrifugation to 70% haematocrit before starting exit experiments by addition of 2.5 ml phosphate-buffered saline (pH 7.4) to 10 μl glucose-loaded erythrocyte

suspension at 0°C. Half-times ($t_{0.5}$) for glucose exit were calculated from first-order logarithmic plots of the loss of cell radioactivity. K_m, V_{max} and inhibition constants were obtained by fitting efflux data to the integrated rate equation of Wharton [7], appropriately modified to include effects of inhibition.

Results and Discussion

Various regions of the human erythrocyte glucose transporter (each about 20–25 amino-acid residues long) were compared with the sequences in the OWL database. The regions chosen were typically those which are relatively well conserved in the various mammalian glucose transporters (GLUT1-GLUT5) [1,3,8–10], and which contain several amino-acid residues capable of forming hydrogen bonds with ligands such as glucose. The searches revealed a number of matches with enzymes involved in carbohydrate metabolism, but surprisingly several sequences showed greater homology with proteins of the NADH-ubiquinone dehydrogenase (ndh) complex than with any other proteins (excluding other glucose transporters) in the data base. Four of the more interesting matches, together with a selection of other matches with carbohydrate-metabolizing enzymes, are shown in Scheme I.

The positions of the various sequences in the human erythrocyte glucose transporter are shown in Fig. 1. It is noteworthy that sequence 4 includes a tryptophan residue (Trp-412) which is a candidate for the site of labelling with the glucose transport inhibitor, cytochalasin B [11,12]. The matches are not restricted to a single chain of ndh, but there is evidence that all the chains of ndh are involved in ubiquinone binding [13] and so the possibility that the homologies reflect common features in the binding sites for glucose and ubiquinone is not excluded.

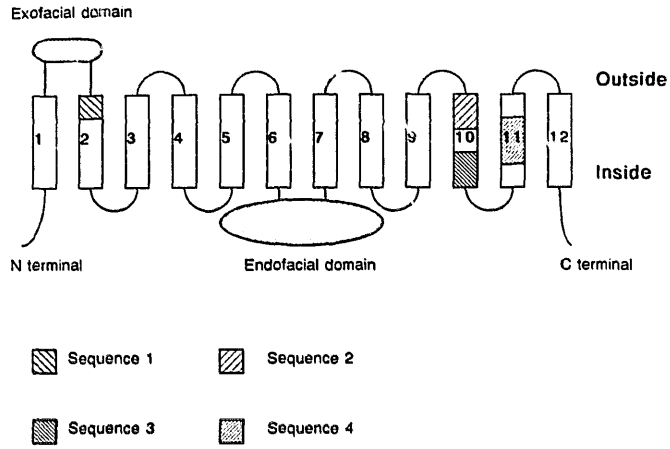

Fig. 1. A diagrammatic representation of the human erythrocyte glucose transporter showing the 12 putative transmembrane segments, the endo- and exofacial domains and the positions of amino-acid sequences 1 to 4.

It is possible that the homologies between the human erythrocyte glucose transporter and ndh simply reflect the fact that both are integral membrane proteins and have little significance in terms of the catalytic properties of the two systems. Alternatively, the homologies could be a signal that the binding sites for glucose and ndh are similar. In keeping with the latter possibility the structures of glucose and ubiquinone (excluding the isoprenoid chain) are similar to the extent that both contain planar (or nearly planar) hexacyclic structures decorated with oxygen-containing attachments capable of receiving hydrogen bonds. Furthermore, when the structures of ubiquinone and glucose are superimposed (Fig. 2) the rms displacement between the oxygen atoms in the two molecules is less than 0.03 nm.

Glucose transport in human red cells is also inhibited by other compounds with hydrophobicities compa-

Sequence 1	
SILPTTLT TLWSLSVAIFSVG	Human erythrocyte, 55–76
NFLPITLAMTLWHISLPISMLG	*Xenopus* ndh chain 1, 281–301
PTSLS TDWSKQSIYF	*Bacillus polymixa*, β-amylase precursor
Sequence 2	
LEQLPWMSYLSIVAIFGFV	Human erythrocyte, 356–376
LMYLAWKSYLPLSLNFLFF	Locust ndh chain 1, 297–319
EQLNWLHYL	*Streptococcus sobrinus*, glucose transferase precursor
Sequence 3	
PIPWFIVAELFSQGPRPA	Human erythrocyte, 384–402
VPKWFIIQELTSQNTTIL	*Xenopus* ndh chain 2, 260–278
PFPWYDVTDNPVDGSNP	Sweet potato, β-amylase
Sequence 4	
NWTSNFIVGMCFQYVEQLCGPYVF	Human erythrocyte, 408–434
TFLMNGYCVMTFYFLTFLCVDYMF	*Leishmania tarolentae* ndh chain 5, 182–208
EWTSNSLIALC	Influenza A, neuraminidase
FFVTLTGQYVPQEDGDYIF	Yeast, β-glucosidase precursor

Scheme I.

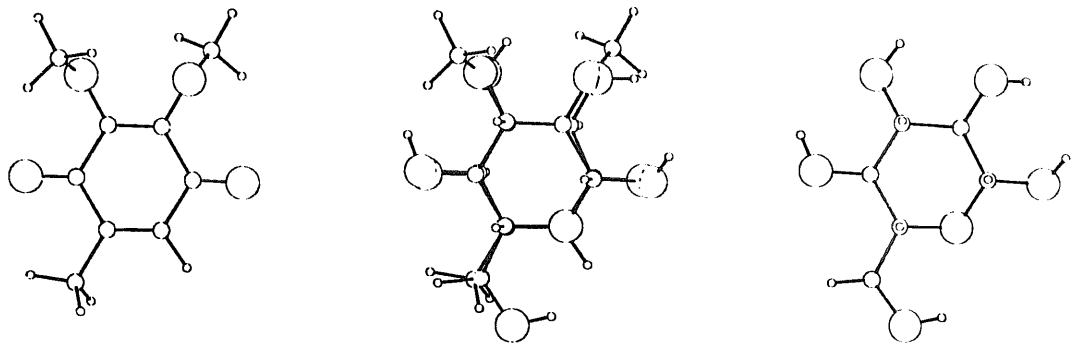

Fig. 2. Energy-minimized representations β-D-glucose (right), ubiquinone Q_0 (left) and the two molecules superimposed (centre).

rable to ubiquinone, including cytochalasin B [11,12], phloretin [14], halogenated pyridines [15] and steroid hormones [16]. This probably indicates that hydrophobicity plays a significant part in the binding of ligands to the glucose transporter, and in this connection it may be significant that one side of the pyranose ring of D-glucose can provide a relatively hydrophobic binding surface.

In view of the above findings we decided to test ubiquinone as an inhibitor of glucose transport in human erythrocytes. Fig. 3 illustrates the inhibitory effect of ubiquinone Q_0 on zero-trans entry of glucose at 0°C, and analysis by non-linear least-squares regression showed that the data give a satisfactory fit to the equation for competitive inhibition between ubiquinone Q_0 and glucose at an external binding site, with a competitive K_i for ubiquinone Q_0 of 0.86 ± 0.20 mM. However, a much better fit can be obtained, with the residual sum of squares decreased 4-fold, if mixed competitive and non-competitive inhibition * is assumed, and non linear regression [18] to the equation,

$$v = (V_{max})/\{(1+([I]/K_{i_2})+(K_m/[S])\cdot([I]/K_{i_1})+([I]/K_{i_2}) + ([I]^2/(K_{i_1}K_{i_2}))\}$$

yields values of 6.1 ± 0.33 μmol l^{-1} s^{-1} for the V_{max} for glucose influx, 0.31 ± 0.040 mM for K_m (glucose), 7.5 ± 3.1 mM for the competitive K_{i_1} for ubiquinone (acting at the exofacial site) and 4.4 ± 0.65 mM for the non-competitive K_{i_2} for ubiquinone (acting at the endofacial site).

Fig 4 illustrates the inhibitory effect of ubiquinone Q_0 on the zero-trans exit of glucose from human erythrocytes. In plots of this type the lines are parallel for competitive inhibition and convergent (meeting on the ordinate) for uncompetitive inhibition. The data cannot be fitted satisfactorily to the model for competitive inhibition, but using the integrated rate equation

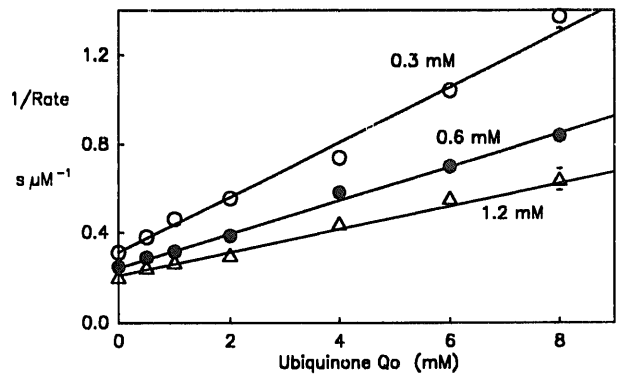

Fig. 3. Inhibition of glucose entry into human erythrocytes by ubiquinone Q_0. Initial rates of influx of [^{14}C]-D-glucose into human erythrocytes measured at 0°C in the presence of 0.3, 0.6 or 1.2 mM D-glucose and the indicated concentrations of ubiquinone Q_0. Points are averages of up to 12 measurements of glucose influx rate and error bars indicate standard errors, which are included within the points where not indicated.

* The analysis of the simple 4-state carrier model by Krupka and Deves [17] shows that inhibition of 'zero-trans' glucose entry or exit by an inhibitor on the trans-side has both non-competitive and uncompetitive components whose relative contributions depend on the ratio

$(1+(h/c)):(1+(h/g))$ for zero-trans entry or

$(1+(g/d)):(1+(g/h))$ for zero-trans exit.

In these ratios c and d are the respective rate constants governing inward and outward reorientations of the loaded transporter, and g and h are the respective rate constants governing inward and outward reorientations of the unloaded transporter. Competition is non-competitive when the ratio is equal to unity, and uncompetitive when the ratio is zero. At 0°C the rate constants have the values $c = 1113\ s^{-1}$, $d = 90.3\ s^{-1}$, $g = 12.1\ s^{-1}$, $h = 0.726\ s^{-1}$ [6], so that trans-inhibition of zero-trans entry of glucose is expected to be almost purely non-competitive (ratio = 1:1.059), whereas trans-inhibition of zero-trans exit is expected to be largely uncompetitive (ratio = 1:15.6).

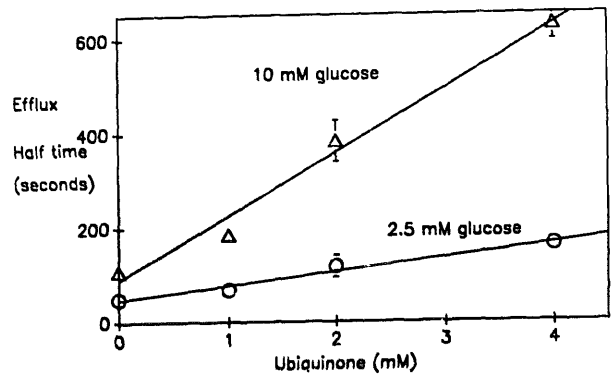

Fig. 4. Inhibition of glucose exit from human erythrocytes by ubiquinone Q_0. Half-times for efflux of [^{14}C]-D-glucose from human erythrocytes were measured at 0°C in the presence and absence of the indicated concentrations of ubiquinone Q_0. Error bars indicate standard errors, which are included within the points where not shown.

for combined competitive and uncompetitive inhibition (see footnote on p. 225),

$$t_{0.5} = ([S]/(2 \cdot V_{max})) \cdot (1 + ([I]/K_{i_c})) + (K_m \cdot \ln 2 / V_{max}) \cdot (1 + ([I]/K_{i_u}))$$

4-parameter, non-linear regression gives the V_{max} for glucose efflux as 73 ± 24 μmol l^{-1} s^{-1}, K_m for glucose as 3.1 ± 2.4 mM, the uncompetitive K_{i_u} for ubiquinone Q_0 (acting at the exofacial site) as 0.47 ± 0.18 mM, and the competitive K_{i_c} for ubiquinone Q_0 (acting at the endofacial site) as 3.8 ± 4.4 mM.

The internal consistency of the above results can be assessed by calculation of the dissociation constants for ubiquinone Q_0 at the exo- and endofacial binding sites of the transporter. As shown in Table I the data for glucose entry and exit agree with one another, yielding dissociation constants of 0.4–0.5 mM at the exofacial side and about 4 mM at the endofacial side of the transporter. The results are therefore consistent with a conventional 4-state transport model in which the affinity of the exofacial site for ubiquinone Q_0 is about 8-times greater than that of the endofacial site. The dissociation constants for ubiquinone Q_0 lie in the same range as those for glucose (10.5 mM) [19] and maltose (0.7 mM) [20] at the exofacial site, and glucose (14.1 mM) [19], propyl β-D-glucopyranoside (8.0 mM) [21], and phenyl β-D-glucopyranside (0.45 mM) [21] at the endofacial site. The affinity of the glucose transporter for ubiquinone Q_0 is thus similar to glucose and its analogues, and ubiquinone Q_0 is one of a number of compounds including phloretin [16] which have a significantly higher affinity for the exofacial than the endofacial binding site.

This finding of two distinct external and internal inhibitory sites for ubiquinone Q_0 lends some weight to the possibility that either or both of sequences 1 and 2 (above) may be involved in the binding of glucose (and ubiquinone) at the exofacial surface of the transporter, while sequence 3 may be part of a second binding site (with lower affinity for ubiquinone) at the endofacial surface.

The amino-acid sequences 1–4 all contain a tryptophan residue which could be involved in substrate binding and, in view of this, we have investigated the effect of the water-soluble tryptophan-reactive agent, dimethyl(2-hydroxy-5-nitrophenyl)sulphonium bromide (DNSB), on glucose influx into human erythrocytes. Table II shows that DNSB did in fact cause concentration-dependent inactivation of glucose transport in an effect that was not significantly altered by pretreatment of the erythrocytes with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in order to protect sulphydryl groups before reaction with DNSB. This excludes the possibility that DNSB-inactivation could be due to reaction with (exofacial) sulphydryl groups [22] and leaves the tryptophan residues in the glucose transporter as the most likely candidates for attack by DNSB. The lack of protection by DTNB contrasts with May's [23] finding that pretreatment of erythrocytes with DTNB completely protected exofacial SH-groups on the glucose transporter from reaction with *S*-(bismaleimidomethyl ether)-L-cysteine. The reactive tryptophan residues are likely to be at the exofacial surface of the glucose transporter since DNSB crosses the cell membrane

TABLE I

Inhibition and dissociation constants for ubiquinone Q_0

Inhibition constants (K_i) for ubiquinone Q_0 were derived from the data in Figs. 1 and 2. Dissociation constants (K_d) were calculated (using the analysis of Krupka and Deves [17] by dividing the inhibition constants by the appropriate conversion factor. Values used for rate constants *c*, *d*, *g* and *h* were, respectively, 1113 s^{-1}, 90.3 s^{-1}, 12.1 s^{-1} and 0.726 s^{-1} [6].

Type of inhibition	K_i (mM)	Conversion factor	Calculated K_d (mM)	
			external	internal
Competitive against glucose entry	7.5 ±3.1	$(1+(g/h)) = 17.7$	0.43±0.18	
Non-competitive against glucose entry	4.4 ±0.65	$(1+(h/c)) = 1.00$		4.4±0.65
Competitive against glucose exit	3.8 ±4.4	$(1+(h/g)) = 1.06$		3.6±4.2
Uncompetitive against glucose exit	0.47±0.18	$(1+(g/d)) = 1.13$	0.42±0.16	

TABLE II

Inactivation of glucose transport by dimethyl(2-hydroxy-5-nitrophenyl)sulphonium bromide

In inactivation experiments washed human erythrocytes at 20–30% haematocrit were incubated with 20 mM dimethyl(2-hydroxy-5-nitrophenyl)sulphonium bromide (DNSB) in phosphate-buffered saline (pH 7.4) for 10 min at 20°C. After this treatment the cells were washed five times with phosphate buffered saline before assay of zero-trans entry of 1.2 mM ^{14}C-labelled D-glucose. In some experiments erythrocytes were pretreated by reaction with 4 mM 5,5'-dithiobis(2-nitrobenzoic acid) for 30 min at 20°C to protect suphydryl groups, before washing and reaction with DNSB. Sulphydryl groups were regenerated by incubation of erythrocytes with 10 volumes phosphate-buffered saline containing 20 mM cysteine for 10 min at 37°C, then cells were washed with phosphate-buffered saline before measurement of [^{14}C]-D-glucose influx. This procedure recovered 84.2 ± 4.3% glucose transport activity. DNSB was dissolved in phosphate-buffered saline containing 1% v/v ethanol (final concentration) immediately before use. The experiments shown describe (a) the concentration-dependence of DNSB-inhibition, and the effects of 10 mM D-glucose and 120 mM maltose when present during treatment with 10 mM DNSB, with no pretreatment (b), or after protection of exofacial SH-groups with DTNB before DNSB-treatment (c). Transport assays were carried out after washing cells free from reagents used in pretreatments. Results (with S.E.) are from sets of three experiments each based on triplicate measurements.

Conditions	Relative glucose transport rate
(a) Control	100
1 mM DNSB	77 ± 11
5 mM DNSB	62 ± 5.1
10 mM DNSB	61 ± 2.9
20 mM DNSB	50 ± 3.6
40 mM DNSB	36 ± 1.8
(b) Control	100
10 mM DNSB	63 ± 3.3
10 mM DNSB + 10 mM glucose	48 ± 5.4
10 mM DNSB + 100 mM maltose	47 ± 10.5
(c) DTNB-treated control	100
10 mM DNSB(DTNB)	61 ± 7.0
10 mM DNSB(DTNB) + 10 mM glucose	70 ± 3.5
10 mM DNSB(DTNB) + 100 mM maltose	50 ± 2.8

relatively slowly, although the possible involvement of highly DNSB-sensitive tryptophan residues at internal site(s) cannot be excluded. Attempts to react DNSB with internally facing tryptophan residues were unsuccessful because exposure of (permeable) haemolysed erythrocyte membranes to DNSB led to agglomeration of the membranes. This agglomeration occurred at concentrations of DNSB as low as 0.1 mM, a results which confirms the low permeability of intact erythrocytes to DNSB.

Table II also shows that extracellular maltose, which binds without being transported and therefore causes accumulation of the externally orientated form of the glucose transporter [20], slightly facilitated inhibition by DNSB. Glucose, which promotes turnover of the carrier and strongly facilitates inactivation by other inhibitors such as 1-fluoro-2,4-dinitrobenzene [24],

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LEQLPWMSYLPTTLT TLWSLSVAIFSVG   Human erythrocyte,
*  * * *l** **l *** l*l * l*     358–367 and 58–75
LMHLVWNSFLPITLAMTLWHISLPISMLG   Xenopus ndh chain 1,
                                  290–318
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Scheme II.

slightly decreased DNSB-inactivation in untreated erythrocytes while increasing inactivation in DTNB-pretreated cells. Measurements of glucose efflux from DNSB-treated erythrocytes are technically difficult because of a tendency of the DNSB-treated cells to lyse during prolonged loading with [^{14}C]-D-glucose. However, results indicate similar extents of inhibition of glucose influx and efflux after treatment of intact erythrocytes with 10 mM DNSB for 10 min at 20°C.

Our finding that ubiquinone Q_0 is an inhibitor of both glucose influx and efflux in human erythrocytes arose directly from attempts to gain insight into the transport mechanism by comparing the glucose transporter GLUT1 with all available protein sequences in the OWL database. Interestingly, sequences 1 and 2 in ndh chain 1 overlap so that, when put together, transmembrane segments 2 and 1 of the glucose transporter match a single stretch of ndh chain 1 from *Xenopus* (see Scheme II).

This raises the intriguing possibility that part of a binding site for ubiquinone, formed by a loop in ndh chain 1, resembles a glucose-binding site formed by the coming together of two parts of the glucose carrier which are distant in the amino-acid sequence. A site of the latter type could be alternately formed and dismantled by changes in conformation of the transporter during the glucose transport process, and this is consistent with the fact that glucose transport kinetics can be described by a 4-state model in which the transporter can present glucose binding sites alternately (but not simultaneously) at the exofacial and endofacial surfaces of the membrane. The results presented here indicate that an exofacial glucose binding site (which may involve amino-acid sequences 1 and 2) binds ubiquinone more strongly than the endofacial site (which may involve sequence 3). Trp-412 has also been identified as a possible target for the site of covalent labelling of the transporter by cytochalasin B [11,12] so that this amino-acid residue, together with others in sequence 4 provide a possible intra-membrane binding site for glucose. All the amino-acid sequences 1–4 will therefore be of interest in any future attempts to probe the mechanism of glucose transport by mutagenesis.

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