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EVIDENCE FOR ALLOSTERIC INHIBITION SITES IN THE GLUCOSE CARRIER OF ERYTHROCYTES

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Summary

2,4-Fluorodinitrobenzene and 2,3-butanedione, which irreversibly inactivate the glucose transfer system of erythrocytes, have been used as probes to determine whether the substrate site and inner and outer sites for reversible inhibitors are located in the same or different regions of the carrier. Inhibitors bound at an inhibition site exposed in the inward-facing but not the outward-facing form of the carrier (cytochalasin B, androstendione and androstandione) protect the transport system against inactivation by 2,4-fluorodinitrobenzene. Inhibitors bound at an external inhibition site (phloretin) and substrates bound at the transfer site do not protect. In contrast inactivation by 2,3-butanedione is slightly accelerated by internally bound inhibitors, while substrates and substrate analogs bound at the transfer site protect the system. It is shown that fluorodinitrobenzene reacts in the inner inhibition site and butanedione in the substrate site; and further that these sites may be separate binding areas in the carrier linked by allosteric interaction. The consequence of this linkage is that binding of a ligand at the substrate site precludes binding of another ligand at the internal or external inhibition site.

Introduction

When a transport system is powerfully but reversibly inhibited by a substance very different from the substrate in structure we are prompted to ask whether the inhibitor may be binding not at the substrate site but at a separate

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allosteric site. Phloretin and cytochalasin B are examples of such inhibitors in the glucose transport system of erythrocytes, for they have an affinity some four orders of magnitude higher than the substrate [1—3].

There is a second reason for suspecting that an allosteric site may exist in this system. Cytochalasin B adds to the inward-facing form of the carrier but not to the outward-facing form [4,5]. Thus, in contrast to the substrate site, which alternates between the two surfaces of the cell membrane, the inhibitor site assumes only one position, suggesting that the sites for the substrate and inhibitor are not tied too closely together. In view of this it is especially remarkable that the inhibition mechanism for cytochalasin B is competitive [4—6].

Studies of peripheral tissues in mammals [7] have indicated that the control of glucose transport is hormonally regulated through the production of an intracellular inhibitor which acts at a site in the inward-facing carrier, and other studies of avian erythrocytes point to a similar conclusion [8]. These various observations suggest that specific regulatory sites are associated with the carrier, which though spatially separated from the substrate transfer site are competitively linked to it by an allosteric mechanism.

Considering the possible regulatory function of the inhibition sites in the erythrocyte system *, their structural relationship to the substrate site is a matter of considerable interest. We have approached this problem by examining the effects of substrates and reversible inhibitors on the inactivation of the system by two chemicals that react covalently with the carrier, namely 2,4-fluorodinitrobenzene [9,10] and 2,3-butanedione [11]. These reagents, when studied in combination, give us a probe to determine whether the substrate and inhibitor sites are in the same or different regions of the carrier. Reaction of fluorodinitrobenzene is greatly accelerated in the presence of good substrates [12], showing that it does not react at the substrate site. It is little affected by phloretin [12] but strongly retarded by cytochalasin B [3]. In contrast, butanedione reaction was reported to be retarded by glucose and accelerated by cytochalasin B, indicating that it may react at the substrate site [11]. The effects of a number of substrates and reversible inhibitors have now been investigated, including inhibitory steroids [13], some of which resemble cytochalasin B in becoming bound almost exclusively at an inner carrier site [14]. All these inhibitors, including phloretin and the steroids, compete with glucose for the carrier [12,14].

Experimental Methods and Results

Human blood was obtained from an outdated blood bank supply. The steroids and cytochalasin B were purchased from Sigma Chemical Co. All chemicals were of commercial reagent grade.

Whole blood was sedimented in a clinical centrifuge and the red cells were washed in isotonic buffer containing NaCl and 20 mM sodium phosphate adjusted to pH 7.5. Following this the cells were washed twice more in an

* It may be noted that regulation of glucose transport, though known in avian red blood cells, has not yet been demonstrated in human red cells.

isotonic solution of NaCl and 30 mM sodium borate buffer, pH 7.7. Packed cells were suspended in this isotonic borate buffer solution (2.5 ml cells in 45 ml of solution), prior to treatment with fluorodinitrobenzene or butanedione. After treatment they were incubated at 37°C in 146 mM glucose for approx. 1 h (10% cell suspension), and the rates of glucose exit were then measured at 25°C by the light-scattering method described by Sen and Widdas [15]. In each experiment rates were determined in at least four separate runs, and the means and S.D. were calculated.

Reaction with 2,4-fluorodinitrobenzene

The suspension of cells in borate buffer (4.75 ml) was treated for 45 min at 25°C in the presence of a reversible inhibitor with 2.03 mM fluorodinitrobenzene, with the addition of a total of 0.25 ml of ethanol. Stock solutions of fluorodinitrobenzene, cytochalasin B and the steroids were all prepared in ethanol. The reaction was stopped by the addition of an equal volume (5 ml) of ice-cold borate buffer, followed immediately by centrifugation. The cells were suspended again in 10 ml of cold buffer, spun, and resuspended in 2.2 ml of 146 mM glucose. From the determinations of glucose flux out of loaded cells, pseudo-first order constants for the rates of inactivation were calculated from the relationship

$$2.3 \log (\tau/\tau_0) = kt \qquad (1)$$

where τ_0 and τ are sugar exit times for untreated cells and cells suspended in fluorodinitrobenzene for t min, respectively. It was previously shown that this equation accurately describes the loss of transport activity over a period of at least 1 h [12]. The ratio of rate constants in the presence (k) and absence (k_0) of an inhibitor or substrate was calculated, together with the S.D. of this ratio. Results for a number of different ligands are given in Table I.

Reaction with 2,3-butanedione

Cells suspended in borate buffer were treated at 37°C for 30 min with 11.1. mM 2,3-butanedione in solutions containing 1% ethanol and an inhibitor,

TABLE I

The ratio of inactivation rates by 2.0 mM 2,4-fluorodinitrobenzene at 25°C with cells suspended in isotonic borate buffer, pH 7.7 (see text), in the presence (k) and absence (k_0) of a substrate or inhibitor. The concentration of the added ligand and its half-saturation constant (Refs. 12 and 14) are also given. The results for glucose and 2-deoxyglucose are taken from Ref. 12, where reaction was in phosphate buffer.

Substrate analog	Half-saturation concentration (mM)	Concentration (mM)	k/k_0
Glucose	2.2	125	2.49 ± 0.22
2-Deoxyglucose	1.8	125	4.74 ± 0.68
Phloretin	$6.6 \cdot 10^{-4}$	$4.0 \cdot 10^{-2}$	0.85 ± 0.075
Corticosterone	0.088	0.88	0.87 ± 0.074
Testosterone	0.043	0.10	0.68 ± 0.071
Androstandione	0.018	0.10	0.38 ± 0.035
Androstendione	0.024	0.10	0.28 ± 0.025
Cytochalasin B	$2.8 \cdot 10^{-4}$	$8.5 \cdot 10^{-3}$	0.12 ± 0.010

TABLE II

The ratio of inactivation rates by 11.1 mM 2,3-butanedione at 37°C with cells suspended in isotonic borate buffer, pH 7.7 (see text), in the presence (k) and absence (k_0) of a substrate or inhibitor. The concentration of the added ligand and its half-saturation constant (Refs. 12 and 14) are listed.

Substrate analog	Half-saturation constant (mM)	Concentration (mM)	k/k_0
Glucose	2.2	125	0.41 ± 0.02
2-Deoxyglucose	1.8	125	0.66 ± 0.03
Xylose	12	125	0.23 ± 0.01
Maltose	14	69	0.33 ± 0.02
1,2-*O*-Isopropylidene glucose	45	129	0.44 ± 0.02
Sucrose	∞	125	1.08 ± 0.02
Phloretin	$5.6 \cdot 10^{-4}$	$4.0 \cdot 10^{-2}$	0.94 ± 0.08
Deoxycorticosterone	0.038	0.20	1.20 ± 0.14
Corticosterone	0.088	0.44	1.11 ± 0.08
Testosterone	0.043	0.10	1.09 ± 0.14
Androstandione	0.018	0.10	1.20 ± 0.07
Androstendione	0.024	0.10	1.13 ± 0.07
Cytochalasin B	$2.8 \cdot 10^{-4}$	$1.0 \cdot 10^{-2}$	1.25 *

* From Ref. 11.

which in its essentials is the procedure followed by Masiak et al. [11]. Where inactivation rates were studied in the presence of a transported sugar, a 5% cell suspension was preincubated for 1 h at 37°C in a solution of 140 mM sugar, in borate buffer, and then treated with butanedione as described above. The reaction was stopped by the addition of an equal volume (5 ml) of ice-cold borate buffer and the sedimented cells were resuspended in glucose solution, prior to incubation and assay of glucose efflux, as described above. As in the case of fluorodinitrobenzene, pseudo-first order rate constants for inactivation were determined from Eqn. 1, and the ratio of rate constants in the presence (k) and absence (k_0) of an inhibitor or substrate were calculated, with the S.D. Results are given in Table II.

Discussion

The site of 2,3-butanedione reaction

All substrate analogs that become bound to the carrier protect the system against reaction with butanedione (Table II). Protection appears to result from steric interference with butanedione at its reaction site, and not from an altered partition of the carrier between conformations that react at different rates with butanedione. The evidence supporting this conclusion is as follows.

Non-transported substrate analogs protect whether they are bound only to the outer form of the carrier, as in the case of maltose, or to both the outer and inner forms, as with 1,2-*O*-isopropylidene-D-glucose. It is to be noted that maltose does not penetrate the cell [16] and is therefore restricted to the external solution; on the other hand 1,2-*O*-isopropylidene-D-glucose penetrates by simple diffusion, as do related compounds [17,18], and adds to the carrier on both sides of the membrane [14]. At a saturating concentration, maltose should therefore draw all the carrier into the outward-facing form, while 1,2-

O-isoporpylidene-D-glucose should allow the outer and inner forms to exist in equilibrium. Phloretin, which like maltose is restricted to the external solution [19], should also draw the carrier into the outer form; phloretin, however, gives no significant protection against butanedione. Cytochalasin B, which is bound only to the inward-facing carrier [4,5] and therefore traps the carrier in this form, likewise offer no protection. It follows that protection by substrates is unrelated to any difference in reactivity of inner and outer carrier conformations, but depends instead on steric interference by ligands bound at the substrate site. Butanedione is therefore likely to react in or near this site.

Though the three substrates, glucose, deoxyglucose and xylose, all protect against butanedione, their effectiveness varies; xylose is better at protecting than glucose, and glucose is better than deoxyglucose. As inactivation rates were seen not to depend on carrier partition between inner and outer forms, it is possible that the substrates offer varying degrees of steric interference in the reaction. If so the reaction site is likely to be near the 2-hydroxyl group and distant from the 6-hydroxymethyl group.

The separation between the sites for substrates and inhibitors

In contrast to substrate analogs, none of the inhibitors structurally unrelated to glucose protects the system against butanedione. Indeed cytochalasin B [11] and steroids (Table II) actually increase the inactivation rate. Cytochalasin B, androstendione and androstandione bind to the inner carrier form and not the outer [4,5,14], which shows that butanedione does not react at the inhibitor binding site on the inner membrane surface. As the inhibitors are bigger molecules than glucose they should offer greater steric protection, were they bound at the substrate site. Hence the inhibition site must be separate. Other steroids, such as corticosterone, testosterone and deoxycorticosterone, add to both inner and outer sites [14]. Phloretin, being restricted to the outer medium, binds to only the external carrier form and has no significant effect on butanedione reaction. It follows that butanedione cannot react at the external binding site either, and that this site, like the internal inhibition site, must lie outside the substrate site.

The site of 2,4-fluorodinitrobenzene reaction

Fluorodinitrobenzene inactivates the carrier more rapidly when good substrates are present [12] and more slowly in the presence of the non-transported analog, maltose [21], which traps the carrier in the outer form. Other glucose derivatives such as 4,6-*O*-ethylidene-D-glucose, which penetrate the membrane passively but which are bound mainly to the outer carrier form, also protect [17,18,22]. On the other hand propyl-β-D-glucose, which passively enters the cell and binds almost exclusively to the inner carrier form, accelerates the fluorodinitrobenzene reaction [23]. These observations on substrates and substrate analogs lead to two conclusions. Firstly, fluorodinitrobenzene reacts outside the substrate site, and secondly its reaction rate depends on the partition of the carrier between different conformational states.

Edwards [24] and Barnett and his coworkers [23] have suggested that the effects of substrates can be understood in terms of a highly reactive inward-facing carrier form and a less reactive outward-facing form. The substrates

which at equilibrium inside and outside the cell accelerate the rate are assumed to shift the carrier distribution in favour of the inner form. This hypothesis is untenable for two reasons. First, the non-transported propylglucoside described above should have a near-maximal effect in raising the inactivation rate, since it was shown to bind almost exclusively to the inner carrier form and should therefore trap nearly all the carrier on the inside [23]. It was observed, however, to be somewhat less effective than glucose, and hence far less effective than 2-deoxyglucose, which is twice as good as glucose [12]. Second, in order for glucose at equilibrium across the cell membrane to increase the amount of the inner carrier form, its affinity on the inside would have to be higher than its affinity on the outside, but the reverse is actually true [25]. The hypothesis must therefore be rejected. Increased inactivation rates are not explained, either, by the reactivity of the outer carrier form, which as noted above is low [21]. If neither the inner nor outer form reacts fast enough to account for the observations, then it is likely that some other carrier species does. This form may be an intermediate between the two surface forms and may accumulate when good substrates are transported [12,20].

Cytochalasin B was shown to provide strong protection against fluorodinitrobenzene (Ref. 3; Table I), and the rate in its presence is much lower than with maltose, which also protects. Cytochalasin B sequesters the carrier in the inner form, but we have seen that this carrier state reacts more rapidly than the undisturbed carrier. The protection by cytochalasin B, therefore, must be steric in orgin. That is, fluorodinitrobenzene probably reacts in or near the cytochalasin B binding site. This conclusion is supported by observations on the binding of cytochalasin B to isolated erythrocyte membranes, which is prevented by reaction with fluorodinitrobenzene [26].

The conclusion is also supported by the protection observed with androstendione and androstandione, which as we have noted are also bound mainly at the inner inhibition site [14]. The protection which they give argues that cytochalasin B and these steroids become bound at the same inner site. Other steroids, corticosterone and testosterone, were shown to bind at both inner and outer inhibition sites [14], and in agreement with this conclusion they are now found to give less protection against fluorodinitrobenzene than those steroids bound mainly at the inner site.

Two classes of reversible competitive inhibitor

From their differing effects on the reactions of butanedione and fluorodinitrobenzene it is clear that inhibitors which compete with the substrate and form a 1 : 1 complex with the carrier fall into two distinct groups. There are on the one hand the substrate analogs, such as maltose, 4,6-*O*-ethylidene glucose and propylglucoside, which are bound at the substrate site, though they do not undergo transport. On the other hand there are compounds unrelated in structure to the substrate, such as cytochalasin B, certain steroids, and phloretin, which are bound at separate inhibition sites. A further division in both these classes may be made into those bound at inner sites (the substrate or the modifier site), and those bound at outer sites.

Interactions between the substrate site and the internal modifier site

Though the inhibition site which binds cytochalasin B, androstendione and

androstandione is outside the substrate site, as seen above, nevertheless the inhibition is purely competitive in mechanism [4—6,14]. The question which we now ask is whether this inhibition is due to partial overlap between the two sites, leading to competition, but allowing for various effects of bound substrates and inhibitors on the reactions of butanedione and fluorodinitrobenzene; or whether the linkage between the sites may be mediated by conformational changes in both sites induced by ligand binding at either. A definite answer cannot yet be given, but the available evidence would favour the second alternative. Aside from the fact that there is no point of structural similarity between glucose and the inhibitors, there is clear evidence in the observations which we have already discussed for linked conformational changes. The acceleration of fluorodinitrobenzene reaction by glucose and deoxyglucose (Table I) shows that substrate binding induces a conformational change at the reaction site, which we now know to be in the cytochalasin B site. Conversely, cytochalasin B [11] and steroids accelerate the reaction of butanedione, implying that they may induce a conformational change in the substrate site, which is where butanedione reacts. Furthermore, in some way which is not yet understood, the inner modifier site plays an essential role in the carrier reorientation step, for it is clear that the reaction of fluorodinitrobenzene cannot prevent substrate binding, but that it does prevent the reorientation step of the carrier-substrate complex.

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