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REFERENCES

Engberg, P., Millqvist, E., Pohl, G., & Lindskog, S. (1985) *Arch. Biochem. Biophys. 241*, 628–638.
Eriksson, A. E. (1989) Doctoral Dissertation, Uppsala University.
Jonsson, B.-H., Steiner, H., & Lindskog, S. (1976) *FEBS Lett. 64*, 310–314.
Kararli, T., & Silverman, D. N. (1985) *J. Biol. Chem. 260*, 3484–3489.
Khalifah, R. G. (1971) *J. Biol. Chem. 246*, 2561–2573.
Kunkel, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
Lloyd, J., McMillan, S., Hopkinson, D., & Edwards, Y. H. (1986) *Gene 41*, 233–239.
Pocker, Y., & Bjorkquist, D. W. (1977) *Biochemistry 16*, 5698–5707.
Pocker, Y., Janic, N., & Miao, C. H. (1986) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., & Zeppezauer, M., Eds.) pp 341–356, Birkhauser, Boston, MA.
Ren, X., Jonsson, B.-H., Millqvist, E., & Lindskog, S. (1988a) *Biochim. Biophys. Acta 953*, 79–85.
Ren, X., Sandstrom, A., & Lindskog, S. (1988b) *Eur. J. Biochem. 173*, 73–78.
Rosenberg, A. H., Lade, B. N., Chui, D.-S., Lin, S.-W., Dunn, J. J., & Studier, F. W. (1987) *Gene 56*, 125–135.
Rowlett, R. S., & Silverman, D. N. (1982) *J. Am. Chem. Soc. 104*, 6737–6741.
Sanyal, G., Swenson, E. R., Pessah, N. I., & Maren, T. H. (1982) *Mol. Pharmacol. 22*, 211–220.
Schowen, K. B. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) Plenum, New York.
Silverman, D. N. (1982) *Methods Enzymol. 87*, 732–752.
Silverman, D. N., & Tu, C. K. (1986) *Biochemistry 25*, 8402–8408.
Silverman, D. N., & Lindskog, S. (1988) *Acc. Chem. Res. 21*, 30–36.
Silverman, D. N., Tu, C. K., Lindskog, S., & Wynns, G. C. (1979) *J. Am. Chem. Soc. 101*, 6734–6740.
Simonsson, I., Jonsson, B.-H., & Lindskog, S. (1979) *Eur. J. Biochem. 93*, 409–417.
Steiner, H., Jonsson, B.-H., & Lindskog, S. (1975) *Eur. J. Biochem. 59*, 253–259.
Tu, C. K., Sanyal, G., Wynns, G. C., & Silverman, D. N. (1983) *J. Biol. Chem. 258*, 8867–8871.
Tu, C. K., Thomas, H. G., Wynns, G. C., & Silverman, D. N. (1986) *J. Biol. Chem. 261*, 10100–10103.
Tu, C. K., Silverman, D. N., Forsman, C., Jonsson, B. H., & Lindskog, S. (1989) *Biochemistry 28*, 7913–7918.
Yagil, G., & Hoberman, H. D. (1969) *Biochemistry 8*, 352–360.

Measurement of Active-Site Homology between Potato and Rabbit Muscle α-Glucan Phosphorylases through Use of a Linear Free Energy Relationship†

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ABSTRACT: The Michaelis–Menten parameters (V_{max} and K_m) for turnover of an extensive series of deoxy and deoxyfluoro derivatives of α-D-glucopyranosyl phosphate by the α-glucan phosphorylase from potato tuber have been determined. Very large rate reductions are observed as a consequence of each substitution, primarily due to losses in specific binding interactions, most likely hydrogen bonding, at the enzymic transition state. Comparison of the V_{max}/K_m values so determined with those measured for rabbit muscle α-glucan phosphorylase [Street et al. (1989) *Biochemistry 28*, 1581] reveals an astonishingly similar specificity, especially in light of the phylogenetic separation of their host organisms. This indicates that very similar hydrogen-bonding interactions between the enzyme and the substrate must be present at the transition states for the two enzymic reactions; therefore, they have very similar active sites. Quantitation of this similarity is achieved by plotting the logarithm of the V_{max}/K_m value for each substrate analogue with the potato enzyme against the same parameter for the muscle enzyme, yielding straight lines (ρ = 0.998 and 0.999) of slope 1.0 and 1.2 for the deoxy and deoxyfluoro substrates, respectively. Since the correlation coefficient of such plots is a direct measure of the similarity of the two transition-state complexes, thus of the enzyme active sites, it can be used as a measure of active-site homology between the two enzymes. The extremely high homology observed in this case is consistent with the observed sequence homology at the active site.

Few methods currently exist for comparing the structures and properties of two similar, possibly evolutionarily related, enzymes and providing an index of their similarity. The most effective and widely used such parameter is the degree of sequence homology derived from comparison of amino acid sequence data. This is a very valuable measure of structural and functional similarity, especially as it can be used to inspect individual parts of the overall sequence in isolation from the remainder. However, it clearly requires prior determination of the amino acid sequence, a nontrivial task. An alternative approach to such comparisons, which would probe similarities in the active-site region, might involve a comparison of the catalytic capabilities of the enzymes in question. This could

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be achieved by measurement of the kinetic parameters for each of the enzymes with a series of conservatively modified substrate analogues, thereby probing the immediate environment of the sites of substitution. If similar changes in rate are observed for the two enzymes as a consequence of a particular modification, then the active-site amino acids interacting with that particular part of the substrate are probably similar in the two enzymes. This could then be quantitated by means of a simple linear free energy relationship in which the logarithm of V_{max}/K_m for each substrate with one enzyme is plotted against the same parameter determined for the other enzyme. The correlation coefficient and, to a certain extent, the slope of the plot would provide a measure of the active-site homologies. This paper describes the successful application of this approach in comparing two α-glucan phosphorylases, that from potato and that from rabbit muscle. These enzymes are ideal for testing this approach since the amino acid sequences of both enzymes are known (Titani et al., 1977; Nakano & Fukui, 1986), since the three-dimensional structure of one of the enzymes is known to high resolution (Sprang & Fletterick, 1979; Sprang et al., 1982; Sansom et al., 1985), and since the requisite extensive series of conservatively modified substrates is available (Withers et al., 1986, 1989).

The α-glucan phosphorylases from potato and rabbit muscle catalyze the reversible phosphorolysis of α-glucans producing glucose-1-P[1] and shortened glucan (Graves & Wang, 1972; Fletterick & Madsen, 1980). The two enzymes are fairly similar in size, the muscle enzyme being 842 amino acids in length and the potato enzyme containing 916 residues. Both contain an essential pyridoxal phosphate coenzyme. They differ, however, in their regulatory properties since the potato enzyme has no regulatory controls, whereas the muscle enzyme is subject to both allosteric and covalent control (Fukui et al., 1982). They also differ in their affinities for the α-glucan substrates. Rabbit muscle phosphorylase binds tightly to highly branched α-glucans such as glycogen and only very poorly to linear glucans, whereas the potato enzyme has high affinities for the linear glucans but not for glycogen (Smith, 1971; Fukui et al., 1982). An understanding of these differences has been obtained through determination of the amino acid sequence of the potato enzyme and comparison of this with the sequence and three-dimensional structure of the muscle enzyme (Nakano & Fukui, 1986). Apart from a 78-residue insertion in the middle of the potato enzyme polypeptide chain, the two enzymes are highly homologous, showing 51% identity of residues. Moreover, the regions of low homology are generally located on the exterior surface of the protein, with the core being much more highly conserved. It is therefore probable that the two enzymes have very similar tertiary structures. The sites on the muscle enzyme that are responsible for covalent and allosteric regulation, the covalent phosphorylation site, the AMP binding site, and the nucleoside binding site, show little or no homology, while the residues at the active site are highly conserved. Further, there is little homology in the glycogen storage site region, and the large 78-residue insertion is located directly adjacent to this. These observations have led to suggestions (Palm et al., 1985; Nakano & Fukui, 1986) that the two enzymes evolved from a common ancestral precursor which functioned simply as a catalyst, much like the current potato enzyme. The mammalian enzyme, however, appears to have diversified through acquiring regulatory properties and the glycogen storage site. This has apparently been achieved without a significant change in the active-site structure.

Considerable insight into the structure of the active site of the muscle enzyme, and the function of the individual amino acids therein, has been provided by the recent kinetic studies involving deoxy and deoxyfluoro analogues of the inhibitor glucose and the substrate glucose-1-P, in conjunction with the latest crystallographic results (Street et al., 1986, 1989; Hajdu et al., 1987). Such deoxy and deoxyfluoro analogues are particularly useful in probing hydrogen-bonding interactions. Both the hydrogen and fluorine substituents are smaller than the original hydroxyl, and thus will not prevent the analogue from binding, while their capacities for interaction with the substrate through hydrogen bonding are quite different. A hydrogen substituent has no significant capacity for hydrogen bonding, while fluorine, which cannot possibly act as a hydrogen-bond donor (proton donor) can, arguably, act as a reasonable hydrogen-bond acceptor. Comparison of binding data for a series of suitably substituted analogues can therefore provide insight into the presence, strength, and polarity of hydrogen bonds at each position. By use of this approach, a "map" of the hydrogen-bonding interactions at the glucose binding site of T-state glycogen phosphorylase was established which agreed well with the crystallographic data and provided useful insight into the strengths of such hydrogen bonds (Street et al., 1986). A similar study has been performed by using the corresponding deoxy and deoxyfluoro glucose-1-P substrates of the enzyme to probe hydrogen-bonding interactions at the transition state (Street et al., 1989). Values of V_{max}/K_m are the most useful in gaining insight into interactions at the transition state since V_{max}/K_m is the apparent second-order rate for the interaction of free enzyme with free substrate and is therefore inversely related to the overall activation free energy ($\Delta G^{\ddagger}$) for the enzymic reaction (Fersht, 1985). Changes in V_{max}/K_m upon substitution of a particular hydroxyl therefore reflect changes in activation free energy for the reaction, and these can be calculated according to

$$\Delta\Delta G^{\ddagger} = RT \ln [(V_{max_1}/K_{m_1})/(V_{max_2}/K_{m_2})]$$

Decreases in rate as a consequence of such substitutions in the case of glycogen phosphorylase were found to be due to a combination of binding and electronic effects, with the former being the dominant factor (Street et al., 1989).

Relative V_{max}/K_m values for such a series of analogues therefore provide a sensitive map of the transition-state structure for that particular enzyme. The values obtained reflect primarily the specific enzyme–substrate binding interactions at the transition state, but also, to some extent, the electronic structure of the transition state. It should therefore be possible to compare transition-state structures for related enzymes by comparison of such sets of data obtained on each enzyme. A quantitative graphical analysis of these data could be achieved through the linear free energy relationship derived by plotting the logarithms of the relative V_{max}/K_m values for one enzyme versus the corresponding value for the other enzyme since this directly compares changes in activation free energy. This paper describes the determination of these parameters for potato α-glucan phosphorylase with an extensive series of modified glucose-1-P analogues and their use in a free energy relationship to compare the active-site structures of the potato and muscle enzymes. Rates of turnover of a series of deoxygenated analogues of glucose-1-P by potato phosphorylase have been measured previously (Zemek et al., 1978); however, the values reported did not correlate at all well with our recent results on the muscle enzyme. This was extremely surprising to us in terms of the high degree of homology of

[1] Abbreviations: glucose-1-P, α-D-glucopyranosyl phosphate; deoxyglucose-1-P, deoxy-α-D-glucopyranosyl phosphate; fluoroglucose-1-P, deoxyfluoro-α-D-glucopyranosyl phosphate.

these two enzymes in the active-site region and required the reexamination described herein. This study was extended considerably by the acquisition of additional data on the fluorinated analogues.

Materials and Methods

The syntheses of bis(cyclohexylammonium) 6-fluoro-, 4-fluoro-, 3-fluoro-, and 2-fluoroglucose-1-P have been described elsewhere, as have syntheses of their deoxy equivalents (Withers et al., 1986, 1989). All other buffer chemicals, substrates, and reagents were obtained from Sigma Chemical Co.

Isolation of potato tuber phosphorylase was performed by a modification of a protocol generously provided by Dr. T. Fukui, itself based upon a published procedure (Kamogawa et al., 1968). The procedure employed is identical with that published up to and including the step involving affinity precipitation of the heat-treated aqueous extract of potato using starch in ethanol. The precipitate obtained from this procedure was then redissolved as described, except that arsenate (5 mM) was included in the mixture to enhance the starch degradation through phosphorylase-catalyzed arsenolysis. This material was then loaded onto a large column (5.0 × 25 cm) of DE52 cellulose equilibrated with 10 mM citrate buffer, pH 6.0, at a flow rate of 400 mL/h and then washed with 10 mM citrate buffer, pH 6.0 (approximately 1000 mL) until the absorbance (280 nm) of the effluent returned to base-line levels. A linear gradient of 0–1 M sodium chloride in 10 mM citrate buffer, pH 6.0, was then applied to the column (2000 mL total volume) at a flow rate of 2 mL/min, and fractions (20 mL) were collected. Fractions were assayed, and those containing phosphorylase were pooled, diluted 3-fold with 10 mM citrate buffer, pH 6.0, to reduce the ionic strength, and loaded directly onto a small column (2.5 × 20 cm) of DE52 cellulose. This column was washed and eluted exactly as described for the previous column, except that a shallower gradient was applied (same eluents, but 2400 mL total volume). Fractions containing phosphorylase were pooled and concentrated to 5 mL by using a Millipore immersible concentrator (30 000 M, cutoff). This solution was then applied to a column (1.6 × 52 cm) of Sephacryl S 200 gel filtration matrix previously equilibrated with a buffer containing 25 mM Tris-HCl/25 mM NaCl, pH 7.5, and eluted with the same buffer at a flow rate of 10 mL/h, collecting 1-mL fractions. Material so prepared was pure by electrophoresis (single band on PAGE) and was stored in this buffer under a toluene vapor.

Values of V_{max} and K_m were determined from the initial rates of saccharide synthesis by monitoring release of inorganic phosphate from the substrate glucose-1-P or its analogue. Phosphate released was measured according to the procedure of Baginski (Baginski et al., 1967), but using 1.5 times the normal level of assay reagents to ensure full color development at high concentrations of phosphate. All reactions were conducted in a buffer containing 40 mM sodium maleate, 250 mM KCl, and 0.13 mM EDTA, pH 6.3. Since the reactivity of the potato phosphorylase toward the different substrates varied greatly, it was necessary to conduct initial range-finding experiments to determine optimum enzyme concentrations and reaction times for each substrate. Typically this involved incubating a single concentration of the substrate (between 20 and 40 mM) with the enzyme and assaying aliquots at different times for phosphate released. Suitable conditions of enzyme concentration and reaction time were chosen from these experiments. In particular, care was taken to ensure that linear progress curves were obtained over these reaction times.

Table I: Kinetic Parameters Determined for Potato α-Glucan Phosphorylase with a Series of Deoxy- and Deoxyfluoro-α-D-glucopyranosyl Phosphates[a]

compd	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (mM)	10^{-4} V_{max}/K_m (μmol·min^{-1}·mg^{-1}/mM)	$\Delta\Delta G^{\ddagger}$ (kcal/mol)
G1P[b]	27 (0.5)[c]	1.5 (0.1)	180 000	
2-fluoro-G1P	8.3 (0.3) × 10^{-4}	1.1 (0.1)	7.6	6.1
3-fluoro-G1P	1.6 (0.1) × 10^{-2}	7.1 (1.1)	22.8	5.4
4-fluoro-G1P	6.9 (0.2) × 10^{-3}	0.6 (0.1)	115	4.4
6-fluoro-G1P	2.2 (0.3) × 10^{-2}	84 (17)	2.6	6.7
3-deoxy-G1P	6.4 (0.7) × 10^{-3}	11 (3)	5.8	6.2
4-deoxy-G1P	0.9 (0.1)	14 (3)	643	3.4
6-deoxy-G1P	5.5 (0.5) × 10^{-2}	51 (7)	10.8	5.9

[a] Parameters determined as described under Materials and Methods. Reaction times and enzyme concentrations used for each substrate were as follows: 2-fluoro-G1P, 750 μg·mL^{-1}, 650 min; 3-fluoro-G1P, 105 μg·mL^{-1}, 525 min; 4-fluoro-G1P, 125 μg·mL^{-1}, 332 min; 6-fluoro-G1P, 125 μg·mL^{-1}, 300 min; 3-deoxy-G1P, 137 μg·mL^{-1}, 360 min; 4-deoxy-G1P, 30 μg·mL^{-1}, 52 min; 6-deoxy-G1P, 124 μg·mL^{-1}, 240 min. [b] G1P = α-D-glucopyranosyl phosphate. [c] Values in parentheses represent the error limits (±) on the reported number.

Control experiments to monitor the spontaneous breakdown of substrate and the stability of the enzyme during these relatively prolonged reaction times were also run. Once reasonable conditions for assaying each substrate had been established, accurate values of V_{max} and K_m were determined from initial rate experiments at seven or eight different substrate concentrations by use of a weighted Lineweaver–Burk analysis (Wilkinson, 1961).

Results and Discussion

Turnover of these modified substrates was extremely slow compared to the parent substrate, necessitating the use of high concentrations of substrate and long reaction times in conjunction with a sensitive phosphate assay. This particular assay also has the advantage of being better suited for use with acid-sensitive phosphate esters, an important point in kinetic studies with deoxy sugar phosphates.

Kinetic parameters determined for potato α-glucan phosphorylase with the series of deoxy- and deoxyfluoro-glucose-1-P derivatives are presented in Table I, along with the errors associated with the determination of each number. Also presented are the values of the increase in activation free energy ($\Delta\Delta G^{\ddagger}$) consequent upon substitution of each hydroxyl by fluorine. These were calculated as described earlier. Clearly the substitution of individual hydroxyl groups around the sugar ring by fluorine or hydrogen has an enormous effect on the rates of their turnover by potato phosphorylase. Values of V_{max} are reduced by factors of from 30 to 30 000, while V_{max}/K_m values are reduced by factors of from 300 to almost 100 000. These results are in complete disagreement with those obtained previously for the deoxy sugar phosphates (Zemek et al., 1978), where the *greatest* reduction in V_{max} value was approximately 11-fold and V_{max}/K_m values were reduced only 70–400-fold. Reasons for these discrepancies are not clear and possibly lie in the different assay procedures employed in the two studies. The previous kinetic studies did not involve measurement of phosphate released, but rather measurement of rates of incorporation of tritium-labeled deoxyglucose units into starch determined by scintillation counting of the starch products. Which assay is inherently the more accurate is not clear. However, the data in Table I are certainly more consistent with expectations based upon sequence homology, as will become apparent shortly.

Presumably these large rate reductions are a consequence of some combination of the loss of specific transition-state binding interactions at each position and intrinsic electronic

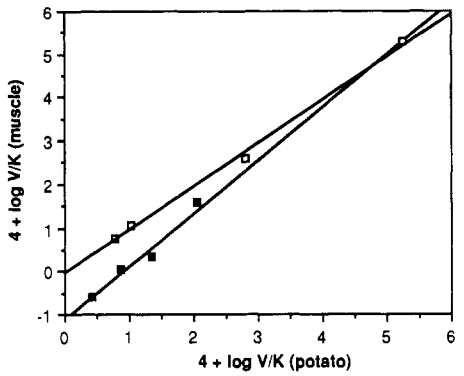

FIGURE 1: Logarithmic plot of the V_{max}/K_m values measured for each of a series of glucose-1-P analogues with rabbit muscle glycogen phosphorylase versus the corresponding parameter measured for the potato α-glucan phosphorylase. Conditions were as described under Materials and Methods. (□) Deoxy sugar; (■) deoxyfluoro sugar.

effects, much as was seen for the rabbit muscle enzyme (Street et al., 1989). Indeed, the magnitude of these effects would appear to be very similar to that observed for the muscle enzyme. Values of $\Delta\Delta G^{\ddagger}$ varied from 3.7 to 8.1 kcal/mol for the muscle enzyme and vary from 3.4 to 6.7 kcal/mol for this potato enzyme. The best means for assessing the similarities of these two sets of data is through the free energy relationship derived by plotting the logarithm of the V_{max}/K_m value of each substrate analogue for one enzyme against the equivalent set of values for the other enzyme. This is, in essence, a direct plot of one set of $\Delta\Delta G^{\ddagger}$ values against another. If the two active sites, thus transition states, are identical, the plot will have a slope of 1 and a correlation coefficient of 1.000. Any differences in types and strengths of hydrogen bonds to individual hydroxyl groups at the transition state would be reflected in scatter of the plot and thus a poorer correlation coefficient. Indeed, since it has been demonstrated (Fersht et al., 1986; Street et al., 1986) that single hydrogen-bonding interactions can contribute up to 4.5 kcal·mol^{-1} to overall interaction energies (equivalent to a 2000-fold rate difference), it is clear that any differences in the polarities or strengths of individual hydrogen bonds will be reflected in large deviations of the individual points from the average line of this plot. Such a plot is presented in Figure 1 and clearly shows that a very strong correlation is present, particularly when the data for the deoxy and deoxyfluoro substrates are plotted separately. Two straight line plots are obtained, one for the deoxy substrates (ρ = 0.999, slope = 1.0) and one for the deoxyfluoro substrates (ρ = 0.998, slope = 1.2). These high correlation coefficients provide *very strong* evidence that the types and strengths of hydrogen bonds to each hydroxyl at the transition state are essentially identical and thus that the active sites are highly conserved.

Since no crystal structure of an active (R-state) glycogen phosphorylase with both substrates bound is yet available, much less the structure of a complex with a good transition-state analogue and the second substrate, it is not possible to unambiguously identify the amino acid residues involved in interactions with each hydroxyl at the transition state. However, a detailed structure of T-state phosphorylase *a* with glucose bound at the active site has been determined and the residues involved in hydrogen bonding have been determined (Sprang & Fletterick, 1979; Withers et al., 1982, 1989). In addition, the structures of several complexes of IMP-activated phosphorylase *b* with substrate analogues have been determined and the interacting residues identified (McLaughlin et al., 1984; Hajdu et al., 1987; Barford et al., 1988). The identities of all the residues found to be interacting with each hydroxyl (with the exception of the 2-position) were found to be essentially identical in all such complexes. It is therefore likely that these same residues are also important in stabilizing the transition state. These residues are listed in Table II, along with their counterparts in the sequence of the potato enzyme as aligned (in the absence of this specific information) by the authors (Nakano & Fukui, 1986) of the sequence paper. As can be seen, all residues, apart from one at the 2-position, are conserved. This is therefore completely consistent with the kinetic parameters reported herein and provides good evidence that this approach to study of active-site homology is a good one. The differences observed at the 2-position are not significant in this study since X-ray crystallographic studies on both phosphorylases *a* and *b* have shown that the binding of substrate glucose-1-P, or analogues thereof, causes a loop of polypeptide (residues 282–286) to be displaced away from the active site and become disordered (Withers et al., 1982; Hajdu et al., 1987; Oikonomakos et al., 1988). It is therefore unlikely that Asn 284 is involved in any interactions with the 2-hydroxyl of glucose-1-P, either in the ground state or in transition-state complexes. Indeed, the observation of a good linear free energy relationship between the two enzymes supports the contention (arrived at from the X-ray crystallographic data) that the residues in this loop are *not* involved in interactions with the substrate. Were this not the case, a large difference in rates for the 2-fluoro-glucose-1-P might be expected for the two enzymes, since a hydrogen bond to a neutral residue in the one case would be replaced by a hydrogen bond to a charged residue in the other.

Table II: Muscle Phosphorylase Amino Acids That Interact with Specific Sugar Hydroxyls and Their Counterparts in Potato Phosphorylase

sugar hydroxyl	muscle phosphorylase	potato phosphorylase
2-hydroxyl	Asn 284 (ND2)	Glu 288
	Tyr 573 (OH)	Tyr 651
3-hydroxyl	Glu 672 (OE2)	Glu 754
	Ser 674 (N)	Ser 756
4-hydroxyl	Asn 484 (OD1)	Asn 561
	Gly 675 (N)	Gly 757
6-hydroxyl	His 377 (ND1)	His 376
	Asn 484 (OD1)	Asn 561

The observation of two lines of different slope for the deoxy and deoxyfluoro substrates, respectively, is particularly intriguing. The origin of the differences in these slopes is not obvious, but is probably a reflection of differences in electronic structure of the transition state rather than differences in binding interactions since it is hard to envisage any mechanism by which differences in binding interactions could be of progressively greater (or lesser) importance in one enzyme versus another, as a function of position of substitution. However, it is quite possible that electronic effects, especially inductive effects, could become progressively less important as the position of substitution becomes more remote from the anomeric center. The data show that the muscle enzyme is more sensitive to fluorine substitution in the substrate than is the potato enzyme. This would suggest a slightly (20%) more cationic transition state for the muscle enzyme than for the potato enzyme. This greater degree of positive charge development presumably reflects a slightly greater extent of bond cleavage at the transition state and thus a slightly more S_N1-like mechanism.

In conclusion, therefore, these data provide strong evidence for the highly conserved nature of the active sites of these two α-glucan phosphorylases, in agreement with the known homologies of the sequences of these two enzymes in the active-site region. The use of a linear free energy relationship to correlate

the specificities of the enzymes from the two sources has allowed quantitation of these similarities through the correlation coefficient and slope obtained. The successful application of this approach here suggests that this may be a useful general method for comparing active sites of two enzymes isolated from different organisms.

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REFERENCES

Baginski, E. S., Foa, P. P., & Zak, B. (1967) *Clin. Chim. Acta 15*, 155.
Barford, D., Schwabe, J. W. R., Oikonomakos, N. G., Acharya, K. R., Hajdu, J., Papageorgiou, A. C., Martin, J. L., Knott, J. C. A., Vasella, A., & Johnson, L. N. (1988) *Biochemistry 27*, 6733.
Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, 2nd ed., Freeman, San Francisco.
Fersht, A. R., Leatherbarrow, R. J., & Wells, T. N. C. (1986) *Trends Biochem. Sci. (Pers. Ed.) 9*, 145.
Fletterick, R. J., & Madsen, N. B. (1980) *Annu. Rev. Biochem. 49*, 31.
Fukui, T., Shimomura, S., & Nakano, K. (1982) *Mol. Cell. Biochem. 42*, 129.
Graves, D. J., & Wang, J. (1972) *Enzymes (3rd Ed.) 7*, 435.
Hajdu, J., Acharya, K. R., Stuart, D. I., McLaughlin, P. J., Bradford, D., Oikonomakos, N. G., Klein, H., & Johnson, L N. (1987) *EMBO J. 6*, 539.
Kamogawa, A., Fukui, T., & Nikuni, Z. (1968) *J. Biochem.* (Tokyo) *63*, 361.
McLaughlin, P. J., Stuart, D. I., Klein, H. W., Oikonomakos, N. G., & Johnson, L. N. (1984) *Biochemistry 23*, 5862.
Nakano, K., & Fukui, T. (1986) *J. Biol. Chem. 261*, 8230.
Nakano, K., Fukui, T., & Matsubara, H. (1980) *J. Biol. Chem. 255*, 9255.
Oikonomakos, N. G., Johnson, L. N., Acharya, K. R., Stuart, D. I., Barford, D., Hajdu, J., Varvill, K. M., Melpidou, A. E., Papageorgiou, T., Graves, D. J., & Palm, D. (1987) *Biochemistry 26*, 8381.
Palm, D., Goerl, R., & Burger, K. J. (1985) *Nature 313*, 500.
Sansom, M. S. P., Stuart, D. I., Acharya, K. R., Hajdu, J., McLaughlin, P. J., & Johnson, L. N. (1985) *J. Mol. Struct. 123*, 3.
Smith, E. E. (1971) *Arch. Biochem. Biophys. 146*, 120.
Sprang, S. R., & Fletterick, R. J. (1979) *J. Mol. Biol. 131*, 523.
Sprang, S. R., Goldsmith, E. J., Fletterick, R. J., Withers, S. G., & Madsen, N. B. (1982) *Biochemistry 21*, 5364.
Street, I. P., Armstrong, C. R., & Withers, S. G. (1986) *Biochemistry 25*, 6021.
Street, I. P., Rupitz, K., & Withers, S. G. (1989) *Biochemistry 28*, 1581.
Titani, K., Koide, A., Hermann, J., Ericsson, L. H., Mumar, S., Wade, R. D., Walsh, K. A., Neurath, H., & Fischer, E. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4762.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324.
Withers, S. G., MacLennan, D. J., & Street, I. P. (1986) *Carbohydr. Res. 154*, 127.
Withers, S. G., Percival, M. D., & Street, I. P. (1989) *Carbohydr. Res. 187*, 43.
Zemek, J., Kucar, S., & Zamocky, J. (1978) *Eur. J. Biochem. 89*, 291.

Comparison of the Toxin Binding Sites of the Nicotinic Acetylcholine Receptor from *Drosophila* to Human†

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ABSTRACT: Recombinant toxin binding proteins have been previously found to provide a convenient experimental system for the study of receptor–ligand recognition (Aronheim et al., 1988). Here, this system has been used to produce the binding sites of the cholinergic receptor derived from seven organisms, *Torpedo californica*, *Xenopus*, chick, mouse, calf, human, and *Drosophila*. These have been compared with respect to their toxin binding capacity. Scatchard analyses show that the K_D values of α-bungarotoxin binding to the above sites are 63, 536, 150, 3200, 6200, 6470, and 1700 nM, respectively. These results reiterate the importance of α183–204 as a ligand binding site. In order to increase the repertoire of sites available for study, chimeric structures were constructed. Through the analysis of such chimeras, some themes of the gross anatomy of the binding site can be learned. A positive subsite followed by a hydrophobic patch preceding a nucleophilic domain appears to be required for efficient toxin binding.

Over the years, numerous receptors have been purified, cloned, and sequenced (Numa et al., 1983; Dohlman et al., 1987; Grenningloh et al., 1987; Schofield et al., 1987; Bunzow et al., 1988; Greve et al., 1989; Mendelsohn et al., 1989; Staunton et al., 1989). However, the nature of the basic recognition process is still unclear. What is the mechanism responsible for ligand binding to its receptor?

In order to address this question, the ligand binding domain of a given receptor must be identified and then analyzed systematically. A case in point is the toxin binding site of the nicotinic acetylcholine receptor (nAChR).[1] Numerous reports have shown that the area of residues 170–210 of the α-subunit

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; BTX, α-bungarotoxin; CTX, α-cobratoxin.

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