Crystallographic Studies on the Structure and Function of Glycogen Phosphorylase b

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Glycogen phosphorylase (EC 2.4.1.1) represents an outstanding example of an enzyme whose activity may be modulated either by non-covalent binding of metabolites or by covalent modification. Phosphorylase b is found in the inactive form in resting muscle; it requires AMP or certain analogues of AMP for activity and is inhibited by ATP, ADP and glucose 6-phosphate. In response to hormonal or nervous stimulation phosphorylase b is converted into phosphorylase a by the action of phosphorylase kinase, which results in the phosphorylation of a single serine residue (Ser-14) per subunit. Phosphorylase a is active in the absence of metabolites, although it is inhibited by glucose. The recent structural results from this laboratory on phosphorylase b (Johnson et al., 1978) and from Edmonton on phosphorylase a (Fletterick et al., 1976; Sygusch et al., 1977) together with the determination of the complete amino acid sequence (Titani et al., 1977) have begun to throw light on the molecular basis for the control and enzymic mechanisms, which are now known to be more intricate than at first supposed.

Methods

The crystals of phosphorylase b are grown in the presence of 2mM-IMP (a nucleotide activator) (Johnson et al., 1974). They are tetragonal, space group P4_12_2 with unit cell dimensions a = b = 12.91 nm, c = 11.59 nm and contain one subunit (mol.wt. 97000) per asymmetric unit. The two subunits of the physiologically active dimer are related by the crystallographic twofold axis at z = 1/4. Data to 0.3 nm resolution were collected on an Arndt-Wonacott oscillation camera for the native crystals and two heavy atom isomorphous derivatives, ethylmercurythiosalicylate and diamino platinum dichloride, and processed as described by Wilson & Yeates (1978).

The path of the polypeptide chain was interpreted from a mini-map (scale 5mm = 0.1 nm) by placing stickers corresponding to a-carbon atoms 0.4 nm apart. Some 801 atoms have been placed, leaving some 40 atoms unplaced out of a total of 841. In a-helical regions and in many areas the chain connectivity could be followed with ease, but in two regions on the interior and one on the exterior of the molecule the connectivity is less certain. The amino acid sequence has been fitted in two long stretches accounting for over half the molecule and work is currently in hand with the fitting of the remainder. While these assignments have still to be confirmed in detail by molecular-model building, the clear densities for certain key residues such as
methionine and those containing aromatic groups give confidence in the present interpretation.

Results and discussion

Structure. The structure is shown in Fig. 1. Briefly, it may be described in terms of three domains: (i) the N-terminal domain, which contains residues 1–310 (upper portion of the molecule in Fig. 1); (ii) the glycogen-binding domain with approximately 160 residues (lower left portion of Fig. 1); (iii) the C-terminal domain with approximately 360 residues (lower right portion of Fig. 1). The overall shape of the subunit is compact apart from the two loops of the N-terminal domain (termed the cap and tower in Fig. 1) which cross the two-fold axis at $z = \frac{1}{2}$ and extend into the symmetry related subunit. The cap region makes contact with the nucleotide-binding site on the symmetry related subunit and is likely to be important in the control properties. The tower extends approximately 1.6 nm into the other subunit and there are extensive interactions between the two symmetry-related towers including a tryptophan–tryptophan interaction with closest approach of approximately 0.4 nm.

The N-terminal domain contains two long $\alpha$-helices, labelled A–A' and B–B' in Fig. 1, which flank the nucleotide binding site. These two helices, appear to be in approximately the same positions as those in phosphorylase a (Fletterick et al., 1976). Similarly there appear to be no differences in the positions of the essential arginine (Li et al., 1977) (Arg-309) on the B–B' helix and the residue (Arg-69) on the A–A' helix, which in phosphorylase a interacts with the phosphate on Ser-14. The most obvious difference between phosphorylase a and b concerns the first 19 residues from the amino terminus. These are flexible in phosphorylase b, but in phosphorylase a (apart from the first eight residues) they appear to be well ordered. Flexible regions have been observed in other proteins and have sometimes been implicated in the conversion of the enzyme from an inactive to active species (for example Fehlhammer et al., 1977). However, the extent to which the localization of the first 19 residues in phosphorylase is correlated with the active conformation of the enzyme remains to be seen, although these residues are apparently important for the control properties. For example solution studies have

Fig. 1. Stereo drawing of the $\alpha$-carbon atoms of one subunit of phosphorylase b

Site N corresponds to the nucleotide allosteric site and the positions of the adenine (Ad) and phosphate moieties (P) of the inhibitor ATP are shown. Site C corresponds to the catalytic site (3.3 nm from site N) with the position of glucose 1-phosphate shown (GIP); 0.7 nm away is the pyridoxal phosphate site (PLP). ATP(2) and site G mark the second nucleotide-binding site and the maltotriose site respectively. The tower and the cap region extend into the symmetry-related subunit of the physiologically active dimer. The cap makes contact to the symmetry related allosteric nucleotide binding site.

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shown that phosphorylase $b'$, a tryptic derivative that lacks the first 16 residues, is active in the presence of AMP, but devoid of homotropic and heterotropic co-operative effects (Graves et al., 1968).

There are two extensive $\beta$-sheet regions in the molecule. One region in the $N$-terminal domain is composed of four parallel and one antiparallel strands and is continued with three further parallel strands in the glycogen-binding domain so that the sheet strands have the topology $1x, 3x, -1, -3x, -1x, -1x$ in the notation of Richardson (1977). The strands in the glycogen domain are connected by $\alpha$-helices so that this structure has the characteristic $\beta$-$\alpha$-$\beta$ fold common in many enzymes, especially those involved in glycolysis. The second sheet region is at the centre of the $C$-terminal domain and comprises six parallel $\beta$-strands and five $\alpha$-helices characteristic of the nucleotide-binding domain first observed in lactate dehydrogenase (Rossmann et al., 1974). In phosphorylase the loop between $\beta$B and $\alpha$C is considerably longer than in lactate dehydrogenase.

**Pyridoxal phosphate**

Pyridoxal phosphate is present in all glycogen phosphorylases studied so far and is essential for activity. Its role, whether structural or catalytic, has long remained a mystery, but current evidence favours a catalytic role for the phosphate (Graves et al., 1978; Feldman et al., 1978). Pyridoxal phosphate is linked via a Schiff base to lysine-679. This residue and the additional electron density representing the cofactor have been identified using the amino acid sequence and the site at which the mercury atom of one of our derivatives binds as a marker. Lysine-679 is located in the 'nucleotide-binding domain' at the $N$-terminal end of the short $\alpha$E-helix. The pyridoxal phosphate is buried in the interior of the molecule, but accessible to the solvent through a narrow crevasse. Its position accords well with predictions made from solution studies, which had suggested a hydrophobic site that is removed from the subunit-subunit interface and that becomes less accessible to solvent on binding glucose 1-phosphate in the presence of an activator.

**Metabolite-binding studies**

Binding studies at 0.3 nm resolution for glucose 1-phosphate, maltotriose and ATP have led to the identification of four major binding sites on the molecule shown in Fig. 1: (i) site N (nucleotide-binding site), which is located in the $N$-terminal domain and which binds the allosteric inhibitor, ATP, tightly and the substrate glucose 1-phosphate weakly; (ii) site C (catalytic site), which is located mostly in the C-terminal domain, but is close to a region where the three domains come together and to the pyridoxal phosphate site (glucose 1-phosphate binds tightly at this site); (iii) a glycogen storage site (site $G$) in the glycogen-binding domain that binds maltotriose; (iv) a second weak binding site for ATP [ATP(2)], which is located at the entrance to the crevasse leading to the catalytic site.

At site N, ATP makes contact with the enzyme through the phosphate groups to basic groups on the B-B' helix. The strand of sheet that carries tyrosine-155 lies underneath this helix and it is possible to see how the AMP analogue, $m$-(m-fluorosulphonylbenzamido)benzylthioadenine could become linked to this tyrosine when bound at site N (Anderson et al., 1973; Titani et al., 1977). The ribose makes contact with the A-A' helix. The adenine moiety is more exposed to the solvent, but it makes contact with part of the symmetry related cap region.

The catalytic site, site C, was identified both from its tight binding of the substrate, glucose 1-phosphate, and from its proximity to the essential pyridoxal phosphate. Glucose 1-phosphate has been fitted to the electron density by using a computer display system. The phosphate is some 0.7 nm from the phosphate of pyridoxal phosphate, but partially shielded from it by a loop of chain from the $N$-terminal domain (residues 130-137). The glucose moiety interacts with residues both from the glycogen-binding domain and the nucleotide-binding domain. There are no large conformational changes on binding the substrate, but small shifts are observed for the loop connecting the tower
to the B–B′ helix. The catalytic site is shielded for the bulk solvent and the only route of access is down the narrow crevasse. This location can thus provide an environment in which phosphorylysis rather than hydrolysis of the α(1–4)-glycosidic bond can be achieved.

At its narrowest point the crevasse has dimensions 0.6 nm × 0.8 nm and is just wide enough to accommodate the second substrate glycogen. However, no binding of the glycogen analogue, maltotriose, has been observed in this region. Instead both in our studies and in those on phosphorylase a (Kavsinsky et al., 1978) maltotriose binds to a site on the surface of the molecule some 3.3 nm from site C and 4.0 nm from site N (Fig. 1). Kinetic studies have shown that phosphorylase activity can be enhanced by pre-incubation with glycogen and the results interpreted in terms of a glycogen-binding site distinct from the catalytic site (Wang et al., 1965). This site seems to be important physiologically for it is possible to isolate from muscle a well-defined structural entity that is rich in glycogen and to which is attached phosphorylase and other enzymes of glycogen metabolism in a protein–glycogen complex (Meyer et al., 1970). Thus we identify the strong maltotriose site as the glycogen-storage site.

The second (weaker) ATP-binding site is at the entrance to the active-site crevasse. The adenine moiety is sandwiched between the long loop between βB and αC of the nucleotide-binding domain and part of the N-terminal domain containing the loop between the bottom of the tower and the B–B′ helix. The phosphates point out into solution and also make contact with part of the glycogen domain. The significance of this site is not understood, although it is worth noting that two nucleotide-binding sites of different strengths have been detected in solution (Wang et al., 1970; Morange et al., 1976).

The structural results clearly show that the strong nucleotide effector site (site N) is some 3.3 nm from the catalytic site (site C). The nucleotide site includes interactions with the symmetry-related subunit. So far in the structural studies on phosphorylase b no obvious conformational changes have been detected which could show how interactions at site N might affect site C. However, the loop of chain involving residues 130–137 and the loop that connects the tower to the B–B′ helix appear to be possible regions through which information might be relayed.