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Regulation of Glucose Metabolism in Bacterial Systems

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In the past 10 years there have been rapid developments in the elucidation of the mechanisms of the Pasteur (or oxygen) and the Crabtree (or glucose repression of the respiratory chain) Effects in bacterial systems, which convincingly exhibit the difference between the regulatory mechanisms in yeast and bacteria. The presented review will demonstrate that the enzyme phosphofructokinase plays no role in the mechanism of the Pasteur effect and that there exists no glucose repression on biomass formation in bacteria under aerobic conditions. Endproduct formation is caused aerobically and anaerobically by an oversupply of $NADH_2$, whereas biomass correlates to energy supply. This development indicates very strongly that the mechanism of the Pasteur effect may be reflected solely in the change of glucose uptake rates and must therefore be sought at the cell membrane. In regard to the Crabtree Effect, the question arises whether there exists such a mechanism in bacteria. The variability of the bacterial electron transport systems, the lack of cytochrome a as terminal oxidase together with bioenergetic investigations indicate that the Crabtree effect may give cause for an alteration but not for a cessation of respiratory activity.

1 Introduction

The regulation of glucose metabolism in facultative anaerobic microorganisms is dominated by two entirely different phenomena, the Pasteur Effect and the Crabtree Effect. The first phenomenon relates to Pasteur's observation [1] that the addition of oxygen reduces the rate of product formation and glucose utilization. Over the past century (see [2]), it has been found that anaerobic growth proceeds with a much smaller energy yield compared with that obtained during respiratory metabolism of glucose to CO_2 and water. In association with the difference in energy formation per mole of glucose degraded, the cell utilizes glucose at a slower rate aerobically in comparison to that consumed during anaerobic growth. This interplay between energy availability and glucose uptake resulted in a number of theoretical concepts of metabolic regulation, but not one has yet been able to account for the Pasteur Effect in its entirety. Most studies have been concerned with the role of the enzyme phosphofructokinase as the regulator of glycolysis, which is reflected in the extensive reviews given of this enzyme in past [3–10].

The second phenomenon relates to Crabtree's observation [11] that respiration rates in tumours grown on glucose were consistently lower than for those grown on xylose. He deduced from his experiments that glycolytic activity must exert a controlling effect on respiration. This phenomenon has since also been found to occur in facultatively anaerobic yeast and bacteria. It is distinct from the Pasteur Effect in that product formation proceeds in the presence of oxygen. Two main theories, both originating in studies using *Saccharomyces cerevisiae* have been proposed as the basis of the Crabtree Effect. The first theory reflects the observations [12, 13] that glucose inhibits the formation of certain enzymes and is a typical example of catabolite repression [14], whereas the second theory [15] suggests that the Crabtree Effect occurs due to a relationship between specific growth rate and metabolic patterns rather than the molecular nature of the substrate.

1.1 Glucose Transport

Glucose added to the medium must cross the cell membrane to be metabolized. Facultative anaerobic bacteria have two dominant systems of glucose transport:

a) the active transport or permease system [182] and

b) the group-translocating phospho*enol*pyruvate-phosphotransferase system (PTS), whereas strictly aerobic bacteria appear to have solely or predominantly the permease system [16, 181].

Investigations into the transport of galactoside [25–27], lactose [28, 29] and alanine [30] and the role of electrical and membrane potentials [31–33] have produced convincing evidence for the existence of the hypothetical chemiosmotic energy-coupling mechanism [34, 35]. Substrate transport and energy production by the bacterial cell are therefore considered in terms of this particular mechanism.

According to Mitchell's theory of chemiosmotic energy-coupling, the permease system [17–20] is regarded as an active transport system which moves molecules (substrate) across the cell membrane against a concentration gradient (for further reading see Ref. [21–24]). The energy required for such active transport system comes

from the membrane-bound ATPase activity, which itself is driven by the proton-motive force created by the aerobic electron transport system (see 1.2). The occurrence of this ATPase activity in the particulate fraction together with the effect of ATP or related energy-rich compounds on this activity led to the suggestion that the permease enzyme may be a membrane-bound galactoside ATPase controlled by the availability of ATP or related compounds such as cyclic AMP.

The group-translocating PTS system, on the other hand, does not require ATPase activity and takes its energy for active transport predominantly from the hydrolysis of phospho*enol*pyruvate or other substrate phosphorylation reactions. The standard free energy of hydrolysis of phospho*enol*pyruvate is about −56.5 kJ (= −13.5 kcal) per mol. The liberated phosphate group is transferred via the PTS components to the substrate, causing a chemical transformation of the substrate molecule (for detailed reading see Ref. [22]), which concomitantly leads to its transport into the cell. Since the approximate values obtainable for the standard free energies of hydrolysis of the other components of the system P-HPr and P-enzyme III are −50.2 kJ (= −12 kcal) per mol, the total free energy available from this system easily substitutes for ATP. This substrate phosphorylation system therefore replaces the oxidative phosphorylation system. Facultative anaerobes could therefore replace the predominant permease system under aerobic conditions with a predominant PTS system under anaerobic conditions.

Apart from its sugar transport, the PTS system also plays a significant physiological role as it regulates the uptake of other carbohydrates [39,40], flagellar synthesis, transmembrane sugar transport, adenylate cyclase (cyclic-AMP synthesizing enzyme) and catabolic enzyme synthesis [41–43]. Of special significance is the effect on adenylate cyclase, since cyclic AMP takes part in the regulation of the synthesis of a number of electron transport chain components, which in turn are responsible for the proton-motive force and thus ATP formation during oxidative phosphorylation. This regulatory system of PTS is itself subject to regulation [44] since the addition of exogenous cyclic AMP reverses the inhibition by PTS on other carbohydrate uptakes provided the exogenous inducers of the particular transport systems are present.

It would appear that cyclic AMP regulates the availability of ATP via oxidative phosphorylation for the active transport or permease system, whereas PTS uses its substrate-level phosphorylation for the group-translocating system. This corresponds with the observations that bacteria utilizing glucose aerobically via the Entner-Doudoroff pathway do not in general posses a PTS system, whereas the latter dominates in those microorganisms, which use exclusively the Embden-Meyerhof-Parnas pathway [44]. It can be envisaged that microorganisms, which switch from one to the other glucose utilizing pathway or using combinations of different pathways could or may have to change from one to the other glucose transport systems depending upon the environmental conditions and particularly the energy status of the cell. Details of the mechanisms responsible for the regulation of carbohydrate uptake in bacteria has been reviewed recently [44].

1.2 Electron Transport Systems

Escherichia coli is able to synthesize a variety of redox carriers depending upon the growth phase, carbon source, strain and growth conditions [34,45–48]. Apart from

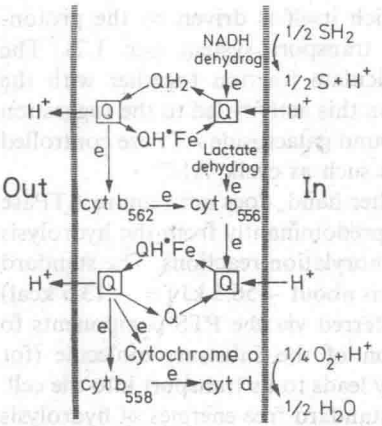

Fig. 1. Electron transport chain of E. coli arranged in the form of the Mitchell proton motive ubiquinone cycle [55]. Abbreviations see Nomenclature. (Reprinted with the permission of the authors and the American Society of Microbiology)

ubiquinone-8, benzoquinone and menaquinone-8, at least nine different cytochromes have been characterized [46,48]. The membrane-bound redox carriers include ubiquinone-8, cytochrome b_{556}, b_{562}, o and d. The latter two serve as terminal oxidases [49–51].

Respiration-driven proton translocation is coupled to the oxidation of NAD(P)-linked or flavin-linked substrates in aerobically grown intact cells of *Escherichia coli*. Stoichiometric measurements of $\rightarrow H^+/O$ ratios provide levels of 4 for L-malate and 2 for the oxidation of succinate and D-lactate [52]. Assuming that 2 H^+ are required per ATP molecule synthesized by ATPase [33], the electron transport chain is considered to be organized into two equivalent conservation segments [53,54], one of which is specifically associated with the NADH dehydrogenase (EC 1.6.99.3) region of the respiratory chain and the other with the cytochrome region [46]. Since each of these segments translocates 2 protons per pair of transferred reducing equivalents, these results indirectly confirm that ATP synthesis via the reversible, proton-translocating ATPase occurs with an $\rightarrow H^+/ATP$ ratio of approximately two. These results are illustrated in Fig. 1 [55] and take into account both the proposed electron transport chain system [56] and Mitchell's proposed protonmotive ubiquinone cycle [57,58].

The relationship between respiratory composition and the efficiency of respiration-linked proton translocation of nine bacterial species of widely differing taxonomic and ecological status has been recently investigated [54]. Efficiencies were found ranging from 4 to 8 mol H^+ per g-atom of oxygen consumed. Clearly, the different patterns of respiratory chain composition [54,59], which are exhibited by those organisms are responsible for the wide variations in proton-translocating efficiency. In *Escherichia coli*, which is a facultative anaerobe, not less than five proton-translocating redox segments have been identified [34]. If each of these segments is considered as a distinct building block, then, dependent on growth conditions, each block can function either separately or sequentially allowing alterations and parallel pathways to exist in the membrane for the reoxidation of reduced coenzymes.

In energy production terms it appears that, under aerobic conditions, the protonmotive force generated by the respiratory chain reverses the ATPase catalysis from ATP hydrolysis to ATP synthesis, which in turn is coupled to inward proton

translocation. Under anaerobic conditions, ATP is gained from substrate-level phosphorylation and a second system functions in the formation of a protonmotive force at the expense of ATP hydrolysis. A third system exists in the form of the group-translocating system, where energy is derived directly by phosphoryl-group transfer from phospho*enol*pyruvate.

2 Effect of Oxygen on Glucose Metabolism (Pasteur Effect)

2.1 Catalytic Reactions of Phosphofructokinase

Phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) catalyzes the transfer of the γ-phosphoryl group of ATP to D(−)-fructose 6-phosphate and produces D(−)-fructose 1,6-bisphosphate and ADP. The enzyme requires Mg^{2+} for the reaction, because $MgATP^{2-}$ is the true substrate [60–62]. The reaction is essentially irreversible and characterized by a large negative value of free enthalpy [63]. Phosphofructokinase (PFK) appears to exhibit little substrate specificity in relation to the phosphoryl donor, with ATP being replaced by all of the nucleotide triphosphates, although greater affinity is shown for the purine nucleotides in bacteria [60, 64, 65]. Unlike the mammalian enzyme, information concerning the substitution of other phosphoryl acceptors has been recorded only in one other instance. In extracts of *Streptococcus faecalis* [66], the PFK-mediated phosphorylation of sedoheptulose 7-phosphate to sedoheptulose 1,7-bisphosphate was recorded.

ATP and citrate are the two major metabolic effectors, which cause inhibition of the mammalian and yeast enzymes [67], but not of the phosphofructokinase from procaryotes. An examination of Table 1 indicates that phosphofructokinase from most bacteria show no response to ATP or citrate. However, there have been some reports that have indicated a response to ATP in the form of inhibition, e.g. *E. coli* [64, 68, 69], *Enterobacter aerogenes* [70], *Clostridium perfringens* and *Staphylococcus aureus* [71]. There is a strong possibility that most of these latter examples are, in fact, allosteric enzymes, which can exhibit an inhibitory effect on the enzyme velocity substrate curves with ATP at low levels of fructose 6-phosphate.

Reports of ATP inhibition were made with Mg^{2+}:ATP^{4-} ratios often less than 2:1, which can indicate either the binding of free ATP^{4-} or a lack of the substrate $MgATP^{2-}$. It is highly improbable that ATP could exist as ATP^{4-} in the intracellular environment, due to the strong chelating ability of the triphosphate group. Blangy et al. [60] made reference to this problem during their detailed kinetic studies of phosphofructokinase in *E. coli* by stating that Mg^{2+}:ATP^{4-} ratios should be at least 10:1 to avoid complex inhibition. As pointed out by Hofmann [4], sigmoidality of the fructose 6-phosphate isotherm is not necessarily the result of the inhibitory action of ATP.

Most of the allosteric bacterial phosphofructokinases are inhibited by phospho*enol*-pyruvate and activated by ADP. There are two exceptions to this rule arising from studies on the allosteric enzymes from *Clostridium pasteurianum* [65] and *Lactobacillus plantarum* [23]. Neither phosphofructokinase from these sources are affected by phospho*enol*pyruvate. The clostridial enzyme was subject only to levels of fructose

Table 1. Action of various effectors on the activities of phosphofructokinase from various sources[a].
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Source	Effectors[b]										
	ATP	ADP	AMP	Pi	PEP	cAMP	Citrate	F 6-P	FBP	NH_4^+	Ref.
Mammalian	I	A	A	A	I	A	I	A	A	A	178)
D. discoideum	NE	I	NE	NE	NE	NE	NE	NE	I	A*	177)
N. crassa	I	I	NE	—	—	—	—	A	—	—	179)
S. cerevisiae	I	NE	A	A	I	NE	I	A	NE	A	82, 174–176)
A. crystallopoietes	NE	NE	NE	—	NE	NE	NE	—	—	NE	73)
B. stearothermophilus	NE	A	—	—	I	—	—	A	—	—	94)
C. pasteurianum	NE	A	NE	—	NE	NE	NE	A	NE	A*	65)
C. perfringens	I	A	A	I	—	NE	—	—	—	—	71)
E. aerogenes	NE	A	NE	NE	—	—	NE	A	—	—	70)
E. coli	NE	A	NE	NE	I	NE	NE	A	NE	A	60)
F. thermophilum	NE	A	NE	NE	I	NE	NE	A	I	—	79)
L. acidophilus	NE	I	NE	—	NE	—	NE	A	A	A	23)
L. casei	NE	NE	NE	A	—	NE	I	A	—	A	72)
L. plantarum	NE	NE	NE	NE	NE	NE	NE	A	NE	A	23, 72)
St. aureus	I	A	A	A	—	A	—	A	—	—	71)
Thermus sp. X-1	NE	A	NE	NE	I	NE	NE	A	—	—	80)

[a] Adapted from Ramaiah [7]; [b] Abbreviations see Sect. 6 Nomenclature
NE = no effect; I = inhibitor; A = activator; A* = absolute necessary for enzyme activity; — = not determined

6-phosphate and the relative concentrations of ATP and ADP. The latter produced a strong activation.

Non-allosteric phosphofructokinases have been reported from a number of bacteria, such as *Lactobacillus casei* [72], *Lactobacillus plantarum* [23, 72], *Arthrobacter crystallopoietes* [73], *Streptococcus faecalis* [74], and *E. coli* [75, 76]. Since the enzymes from these sources do not have a sigmoidal rate dependence on fructose 6-phosphate, they may not have a regulatory role in glucose metabolism. As an example, it was shown [77] that *L. casei* has an allosteric lactate dehydrogenase subject to fructose 1,6-bisphosphate activation and ATP inhibition, which displaces the pivotal role of phosphofructokinase in this organism to lactate dehydrogenase.

2.2 Allosteric Phosphofructokinase

2.2.1 Action of Effectors an Allostery

Most bacterial phosphofructokinases are allosteric due to the cooperative subunit interactions that occur in the presence of fructose 6-phosphate. An important aspect of allosteric enzymes is their response to a number of metabolic effectors. In order to analyze this response in a quantitative manner, investigators make use of the Hill equation as applied to the allosteric concept of Monod et al. [78]. From this approach a useful measure of subunit interactions, or cooperativity is given by the interaction coefficient, n_H (Fig. 2).

The interaction coefficient is affected differently by various effectors. The action of the allosteric effectors ADP and phospho*enol*pyruvate on the *E. coli* enzyme weakens considerably the kinetic interaction of fructose 6-phosphate [60]. ADP is a positive effector, shifting the fructose 6-phosphate velocity curve to the left, decreasing the n_H value from 3.8 to a level of about 1.0. In fact, ADP not only influences the cooperativity of several bacterial phosphofructokinases in the same manner, it also appears to act as a competitive inhibitor of ATP for the enzyme [60, 65, 70, 79]. Phospho*enol*pyruvate, on the other hand, acts in a converse manner by shifting the fructose 6-phosphate velocity curve to the right, and increasing the n_H value to 4.0. This is an example of a negative effector, and results in a low affinity of phosphofructokinase for its substrate fructose 6-phosphate. Amongst the lactic acid bacteria only the phosphofructokinase of *Lactobacillus acidophilus* shows allostery. This enzyme is activated by fructose 1,6-bisphosphate [23]. ADP does not influence the value of the interaction coefficient, but fructose 1,6-

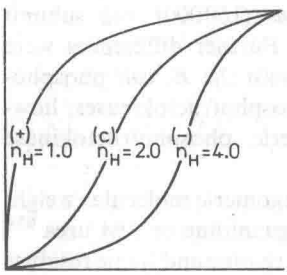

Fig. 2. Diagrammatic Hill plot

bisphosphate has an effect by decreasing the n_H value to 0.8 and shifting the fructose 6-phosphate isotherm to the left. NH_4^+ ion activation also appears to change the interaction coefficient of the *L. acidophilus* enzyme, with a reduction from 3.3 to 2.2. The extreme thermophile, *Thermus sp.* X-1 possesses a phosphofructokinase, which is analogous to the *E. coli* enzyme in its response to metabolite effectors [80].

The pH of the medium plays an important role in the ATP inhibition of phosphofructokinase and implicates the hydrogen ion as a metabolic effector. Results with the yeast enzyme show that an increase of the pH value from 6 to 8 shifts the sigmoidal fructose 6-phosphate velocity curve to the right, with a corresponding increase in the inhibitory action of ATP. At pH 6.0, ATP is less inhibitory and fructose 6-phosphate possesses a higher affinity for the enzyme than at higher pH values [81–83]. Amongst the bacterial sources of phosphofructokinase, only the allosteric enzyme from *L. acidophilus* shows any pH effect [23]. At pH 8.5, the saturation curve for fructose 6-phosphate is markedly sigmoidal in the presence of ATP, with an n_H value of 2.5. Sigmoidality is reduced at low pH values to an n_H value of 1.1 at pH 6.0. Since the grade of cooperativity of fructose 6-phosphate is affected by the pH of the assay medium, and increases at alkaline pH, it is interesting to reflect on the versatility of the enzyme in *L. acidophilus*. The normal environment for the lactic acid group of bacteria is acidic, and phosphofructokinase is non-allosteric at pH 6.0, so the intracellular pH may have a regulatory effect on metabolism. *L. acidophilus* is also found in the alkaline intestinal environment, and could be expected to behave similarly to the allosteric enzyme from *E. coli*, which does not show any sigmoidal change below pH 8.0 [60].

2.2.2 Structure of the Enzyme

In contrast to the phosphofructokinase from yeast [84–92], the prokaryotic enzyme appears to be considerably smaller with an oligomeric molecular weight of about 140000, consisting of four identical subunits of about 35000 [23, 49, 60, 93–96].

Phosphofructokinase from the lactic acid bacteria, *L. plantarum* and *L. acidophilus*, appear to be identical in size, each with a molecular weight of approximately 150000, consisting of four subunits of 38000. Although these enzymes differ kinetically, they do appear to be immunologically similar [23]. This could suggest similarities with regards to the spatial arrangements of the peptide chains, and in regions of amino acid composition. However, no information on the chemical composition of the two lactic acid bacterial phosphofructokinases are available.

Species of enterobacteria behave differently from the lactic acid bacteria [97]. Although the phosphofructokinases consist of tetrameric proteins of four equal subunits, the molecular weights vary between *E. coli* (140000), *Hafnia alvei* (140000), *Enterobacter aerogenes* (110000) and *Serratia marcescens* (160000) and subunit values of 35000, 35000, 32000 and 39000, respectively. Further differences were observed in the heat stability and unequal inactivation with the *E. coli* phosphofructokinase antisera during immunoprecipitation. All phosphofructokinases, however, cross-react immunologically with *E. coli* allosteric phosphofructokinase antisera [97, 98].

Clostridium pasteurianum phosphofructokinase has an oligomeric molecular weight of 144000 and is dissociated to subunits of 35000 in 7 M guanidine or 8 M urea [65]. The amino acid analysis shows that the enzyme contains 34 arginine and lysine residues

Table 2. The amino acid composition of *Escherichia coli*, *Clostridium pasteurianum* and *Thermus sp.* X-1 phosphofructokinases [80].
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Category	Number of residues		
	Thermus sp. X-1	*E. coli*	*C. pasteurianum*
Basic residues	145	140	133
Arginine	74	84	70
Lysine	71	56	63
Acidic residues	227	254	220
Aspartate	109	133	108
Glutamate	118	121	112
Ambivalent residues	324	346	269
Alanine	108	120	92
Half-cysine	12	26	17
Histidine	40	27	10
Serine	52	61	58
Threonine	76	63	70
Tryptophan	4	4	0
Tyrosine	32	45	22
Hydrophobic residues	394	449	405
Isoleucine	109	116	102
Leucine	90	101	103
Methionine	20	44	54
Phenylalanine	30	42	31
Proline	35	40	24
Valine	110	106	91
Glycine	169	157	151
Hydrophobicity (cal/residue)	1043	1091	1057
Hydrogen bonding residues (%)	33	35	34

per 35000. Tryptic peptide mapping revealed 35–85 peptides, and 15 arginine-containing peptides were observed, which is in close agreement with 18 arginine residues per 35000. These results suggest that the clostridial phosphofructokinase is a tetramer consisting of four identical subunits.

Homogenous *Thermus sp.* X-1 phosphofructokinase has a molecular weight of 132000 and this molecular weight decreased to 34000 in 6 M guanidine [80]. In a comparative study of the enzyme from the extreme thermophile with phosphofructokinase from the mesophile *E. coli* and *Cl. pasteurianum*, Cass and Stellwagen [80] use the amino acid composition of the respective enzymes to determine the reason for the extreme heat stability of the *Thermus sp.* X-1 phosphofructokinase. The amino acid composition (Table 2) indicates that the hydrophobicity and the contents of potential hydrogen bonding residues do not differ to any significant degree. The amino acid composition grouped as acidic, basic and hydrophobic, or ambivalent classes of residues are very similar, varying by no more than 3 mol% for a given class among all the enzymes. These results predict a high degree of sequential and structural homology, and suggest that a small number of amino acid differences can account for substantial differences in structural stability. Clearly hydrogen binding alone cannot account for the additional stability in the phosphofructokinase from

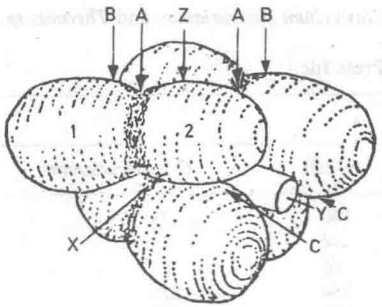

Fig. 3. Schematic drawing of Bacillus stearothermophilus phosphofructokinase [99]. Each subunit is shown divided into two domains, numbered 1 and 2. The four subunits are related by three (X, Y, Z) orthogonal dyad axes (D_2 symmetry). The positions of some of the sites A, B and C are marked, showing that sites A and C lie between subunits. Dark shaded bands are regions of β-sheeting. (Reprinted with the permission of the authors and from Macmillan Journals Ltd.)

the extreme thermophile. Although it has been calculated [51] that only 5–10 kJ per mol of additional free energy is required to stabilize an enzyme structure from 37 to 60 °C, which could be provided by hydrogen bonds or salt bridges.

The structure of phosphofructokinase from *Bacillus stearothermophilus* originally investigated by Hengartner and Harris [94] and Hudson et al. [67], was resolved into a tetrameric protein of molecular weight 136000 with a subunit molecular weight of 33900. A very recent and elegant study on the orthorhombic crystals of the purified enzyme makes use of X-ray diffraction and electron density mapping procedures to obtain a complete structural determination [99]. The enzyme consists of two domains (Fig. 3), numbered 1 and 2, each of which has a central β-pleated sheet sandwiched between α-helices. Each subunit forms contacts with only two of the other subunits in the tetramer. All of the subunits are related by three orthogonal dyad axes (I_{222} symmetry), which coincides with the crystallographic symmetry axes. Apparently there is a solvent filled hole of approximately 7 Å diamter through the center of the tetramer along the Y-axis, indicated by a cylinder in Fig. 3. The positions of some of the sites A and B (active sites) and C (effector site) are marked. Sites A and C were found to lie between subunits and Table 3 indicates the compounds found to bind these sites. An interesting finding is that each subunit in the tetramer has an interface with two other subunits, one of these being bridged by fructose 6-phosphate, the other by the effectors. In addition, the region between the active site and the effector site contained amino acid side chains pointing in one direction to the fructose 6-phosphate site and in the opposite direction to the effector site. Binding of a ligand to either side, therefore, could affect the ligand binding affinity in the other site, which does indicate that both catalysis and control require the whole tetramer.

2.2.3 Kinetic Modelling of the Reaction Mechanism

A reaction mechanism is extremely difficult to examine and to explain if some metabolites have the ability to act as activators or inhibitors. It was therefore necessary to establish an adequate model to explain the mechanism of sigmoidal kinetics. The first model was a concerted symmetry model [78], which assumes that the enzyme phosphofructokinase exists in two different conformational states, R and T, considered to be in equilibrium (Fig. 4). The kinetic data fit a model in which two states have equal affinity for ATP but differ with respect to their affinity for fructose 6-phosphate, ADP and phospho*enol*pyruvate. Although this concerted symmetry model turned

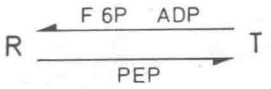

Fig. 4. Two state symmetry model of the reaction mechanism of E. coli phosphofructokinase [78]

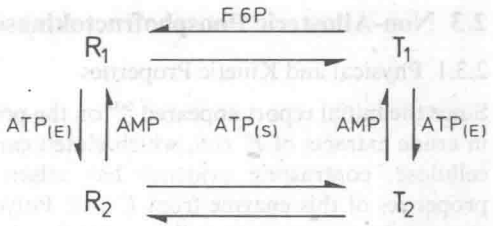

Fig. 5. Four state kinetic model of the reaction mechanism of yeast phosphofructokinase [100]. (Reprinted with permission of the authors and Elsevier (North Holland Biomedical Press))

out to be appropriate for the phosphofructokinases of *E. coli* [60], *Clostridium pasteurianum* [65], *Thermus sp.* X-1 [80] and *Bacillus stearothermophilus* [67], it could not explain the allosteric kinetics of the enzyme from *Lactobacillus acidophilus* [23]. The main problem was the dual action of fructose 1,6-bisphosphate as an activator and as a negative effector. Since the phosphofructokinase of *E. coli* exhibits an analogous problem with respect to the binding of ADP, it is not clear why these phenomena cannot be incorporated into Monod's model.

The other alternative to the concerted symmetry model is the four state kinetic model of yeast phosphofructokinase [100], which involves conformational adjustments of the enzyme. In this model (Fig. 5) the basic conformational states, R and T, are degenerated into two isomeric subconformations, R_2 and T_2. Fructose 6-phosphate binds only R_1 and T_1 with different affinities, but not at all to R_2 and T_2. ATP as a substrate binds to all four states with equal affinity. The inhibitor ATP_E, however, binds only to R_2 and T_2 with the same affinity. Inversely, the activator or positive effector AMP has the same affinity for only the R_1 and T_2 states. In this model then, cooperativity from fructose 6-phosphate is generated by shifting the equilibrium between T_1 and R_1. Therefore, the action of ATP_E merely shifts the saturation curve for fructose 6-phosphate to the right, and that of AMP to the left without any effect on the equilibrium between R_1 and T_2. Consequently, the cooperative interaction of fructose 6-phosphate is not affected by these effectors. This model does not include, however, the complex actions of ADP, which not only inhibits the enzyme in competition with ATP, but also activates it synergistically with AMP [68, 82].

A modification of this model has been suggested by Laurent et al. [101] after demonstrating that the inhibitory action of ATP occurred over a much larger range of ATP concentrations (10^{-6} to 10^{-3} M) than predicted by a concerted transition. Their results, utilizing fluorescent stopped flow and titrimetric measurements, was consistent with a concerted transition of the enzyme in the presence of fructose 6-phosphate, but the regulation by the adenylates appeared to involve a more complex mechanism involving non-concerted, sequential and conformational adjustments of the enzyme.

At the moment there are clear indications, that the bacterial phosphofructokinase mechanism, although not as complex as that of the yeast enzyme, can be explained by the Monod model.

2.3 Non-Allosteric Phosphofructokinase

2.3.1 Physical and Kinetic Properties

Since the initial report appeared [60] on the presence of a phosphofructokinase activity in crude extracts of *E. coli*, which eluted earlier than the allosteric form on DEAE-cellulose, contrasting evidence has arisen concerning the physical and kinetic properties of this enzyme from *E. coli*. Polyacrylamide gel studies on crude extracts showed the enzyme to be a dimer of molecular weight 180000 [64]. Kotlarz and Buc [95], who first succeeded in the isolation and purification of the enzyme from the original mutant strain of Morrissey and Fraenkel [102], reported the presence of a dimeric molecule with a molecular weight of 71000. A dimeric protein of 65–67000 molecular weight has also been isolated from the wild-type *E. coli* K-12 [103, 104]. In contrast to these results, Babul [105] isolated a tetrameric protein with a molecular weight of 140000 from the original mutant strain. Recent work in our laboratory [106] verified the presence of a tetrameric non-allosteric phosphofructokinase in crude extracts of the wild-type *E. coli* K-12. It became apparent that the discrepancies were due to the properties of a facile protein and that molecular size studies utilizing polyacrylamide gels and crude enzyme extracts with associated protein aggregation can lead to erroneous conclusions [98].

The non-allosteric phosphofructokinase from *L. plantarum* is a tetramer possessing identical subunits each of 38000 molecular weight [23].

In its dimeric form, the *E. coli* enzyme has an isoelectric point (pI) of 5.1 at 4 °C and a calculated half-time ($t_{1/2}$) of inactivation at 50 °C of 2.49 minutes, in comparison to a pI of 5.2 and a $t_{1/2}$ of 1.7 minutes at 75 °C for the allosteric isozyme.

ATP inhibition was originally considered to be a regulatory phenomenon with all phosphofructokinases, including that of *E. coli*.[68, 69]. Early attempts characterized the two isozymes in *E. coli* as an 'ATP-insensitive' dimer and an 'ATP-sensitive' tetramer [64, 107]. Detailed kinetic studies have shown that ATP does not inhibit the *E. coli* enzymes [60, 76, 95, 104]. Therefore the two phosphofructokinase isozymes would be more correctly labelled as allosteric and non-allosteric with regards to the second substrate fructose 6-phosphate.

The non-allosteric isozyme can utilize tagatose 6-phosphate at approximately 40% of the normal forward reaction rate obtainable with fructose 6-phosphate [105]. There is also a tendency to utilize a variety of nucleotide triphosphates in substitution for ATP with little specificity between the purine or pyrimidine base [104]. This property is particularly evident with Mn^{2+} and to a lesser degree in the presence of Mg^{2+}. The V_{max} values for ATP in the presence of Mn^{2+} are 2-fold greater in magnitude than in the presence of Mg^{2+}, indicating a Mn^{2+}-mediated activation of the enzyme. Free manganese also decreases the K_m for fructose 6-phosphate. In addition, the rate of catalysis is dependent on the $MnATP^{2-}$ complex and not on free Mn^{2+}. An interpretation of the kinetic data indicated that manganese complexed as $MnATP^{2-}$ was a prerequisite for enhanced catalysis, and by comparison a more efficient substrate than $MgATP^{2-}$. Although this is the first evidence of a manganese-mediated activation for a bacterial phosphofructokinase, it will not be possible to conclude a positive binding of free Mn^{2+} to the enzyme without an accurate assessment of possible enzyme-Mn^{2+} ternary binding complexes. A recent study has demonstrated an activation of yeast phosphofructokinase by $MnATP^{2-}$ [108].