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Cooperativity in Human Erythrocyte Phosphofructokinase†

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ABSTRACT: A kinetic study of purified phosphofructokinase has revealed intermediary plateau regions in substrate saturation curves for fructose 6-phosphate and ATP at the optimal pH, 8.4. At pH 6.8 similar behavior was seen with one substrate, fructose 6-phosphate, but not ATP. Cyclic 3′,5′-AMP and AMP activated the enzyme at pH 6.8 but not 8.4. At the lower pH cyclic 3′,5′-AMP eliminated the intermediary plateau region, producing simple substrate saturation curves. Photooxidation treatment of the enzyme with Methylene Blue abolished cyclic 3′,5′-AMP activation, and also abolished the plateau regions at either pH. The plateau regions and cyclic 3′,5′-AMP activation are therefore likely to depend on closely linked mechanisms. On the other hand, inhibition by high concentrations of ATP is apparently an independent phenomenon, since it persists after photooxidation treatment. In both cases where the plateau regions were eliminated—with cyclic 3′,5′-AMP and after photooxidation—the new K_m was intermediate between the two apparent K_m values of the unmodified enzyme. This circumstance suggests a mechanism involving several sites which exhibit positive cooperativity and possibly negative cooperativity as well, rather than two or more independent sites.

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) catalyzes the nearly irreversible phosphorylation of fructose 6-phosphate by ATP to form fructose 1,6-bisphosphate ($FruP_2$)[1] and ADP. It is recognized as a key enzyme in the regulation of glycolysis (Newsholme and Randle, 1961; Lowry *et al.*, 1964; Vinuela *et al.*, 1963). Phosphofructokinase isolated from heart (Mansour, 1965), brain (Passoneau and Lowry, 1963), muscle (Ling *et al.*, 1965), yeast (Ramaiah *et al.*, 1964), *Escherichia coli* (Atkinson and Walton, 1965), and the liver fluke (Mansour and Mansour, 1962) have similar characteristics. All are inhibited by high concentrations of ATP and the structurally unrelated compound, citrate. The inhibitions of the enzyme by ATP and citrate are reduced in the presence of NH_4^+, orthophosphate, FruP, and 5′-AMP.

Mansour and Mansour (1962) first reported an activation of the enzyme by cyclic 3′,5′-AMP (using the enzyme from liver fluke). A catalytically active form of the enzyme from guinea pig heart and a smaller, catalytically inactive form, possibly a monomer, were later demonstrated in sedimentation experiments (Mansour, 1965). The "inactive" form regained activity at slightly alkaline pH and in the presence of ADP, FruP, or $FruP_2$. With crystalline sheep heart phosphofructokinase, Mansour *et al.* (1966) and Mansour and Ahlfors (1968) suggested that regulation of enzyme activity depended on a

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[1] Abbreviations used are: FruP, fructose 6-phosphate; $FruP_2$, fructose 1,6-bisphosphate; *n*, interaction coefficient in the Hill equation.

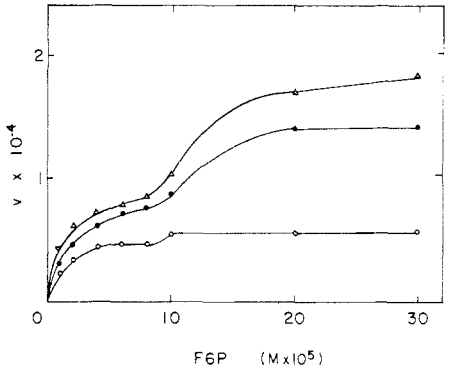

FIGURE 1: Plot of the initial reaction velocity *vs.* the molar concentration of FruP at fixed ATP concentrations at pH 8.4. FruP concentration was varied between 1×10^{-5} and 3×10^{-4} M. Velocities are expressed as nmol of $FruP_2$ formed/10 min per ml of enzyme. Other assay conditions as described in the text: (○) 1×10^{-5} M ATP; (●) 4×10^{-6} M ATP; (△) 8×10^{-5} M ATP.

reversible dissociation of a fully active polymeric form of the enzyme to partially active dimers.

A characteristic of the enzyme isolated from a variety of sources is the sigmoidal dependence of the velocity when FruP is varied and the other substrate, ATP, is present at inhibitory levels (Vinuela *et al.*, 1963; Passonneau and Lowry, 1963; Ramaiah *et al.*, 1964; Mansour, 1963; Atkinson and Walton, 1965; Atkinson *et al.*, 1965; Mansour and Ahlfors, 1968). Activators convert the sigmoidal curves into hyperbolas by antagonizing the inhibitory effect of ATP, possibly by weakening the binding of ATP at an allosteric site (Kemp and Krebs, 1967).

Human erythrocyte phosphofructokinase had received little attention until a new human disease was described, characterized by a deficiency of muscle phosphofructokinase (Tarui *et al.*, 1965; Layzer *et al.*, 1967). A partial purification of the enzymes from human muscle and erythrocytes has been reported (Layzer *et al.*, 1969). The two enzymes differed in molecular weight, electrophoretic mobility, and some kinetic properties. Specifically, erythrocyte enzyme was more susceptible to ATP inhibition and less to citrate inhibition than muscle enzyme (Layzer *et al.*, 1969). Phosphofructokinase from human erythrocytes and the enzyme isolated from rat erythrocytes (Kuhn *et al.*, 1969) show similar properties.

A kinetic study of human erythrocyte phosphofructokinase at pH 8.4 and 6.8 is described below, as well as similar studies following photooxidation treatment of the enzyme. The observations differ significantly from those in two other reports (Staal *et al.*, 1972; Layzer *et al.*, 1969). These discrepancies could be attributable either to different procedures for enzyme purification (Layzer *et al.*, 1979; Lee, 1972; Wenzel *et al.*, 1972; Staal *et al.*, 1972) or to different conditions of assay.

Materials and Methods

Enzyme Purification. Fresh heparinized blood was collected and the erythrocytes were washed and hemolyzed by the addition of an equal volume of water. Phosphofructokinase in the hemolysate was isolated and purified by DEAE-cellulose chromatography and ammonium sulfate fractionation as described in a previous paper (Lee, 1972).

Measurement of Enzyme Activity. Enzymatic activity was measured by a modification of the procedure of Blanchaer *et al.* (1955) for phosphofructokinase in hemolysates in which the enzyme was rate limiting in a coupled sequence of reactions leading to the reduction of NAD^+ by glyceraldehyde-3-phosphate dehydrogenase. The resultant increase in optical density at 340 mμ was followed for 10 min in a recording Gilford Model 2000 Beckman DU spectrophotometer through a light path of 1 cm at 37°. Initial reaction velocity was calculated as nanomoles of $FruP_2$ formed per minute per milliliter of enzyme preparation using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} for NADH (Horecker and Kornberg, 1948). One milliliter of enzyme normally contained 1–2 mg of protein. Two reaction mixtures were used in the kinetic studies.

REACTION MIXTURE A. The following reagents were contained in a final volume of 3.5 ml: 57.0 mM Tris buffer, pH 9.0; 28.5 mM sodium arsenate; 28.5 mM sodium phosphate buffer, pH 7.4; 34.0 mM cysteine; 3.4 mM magnesium chloride; 1.4 mM FruP; 0.35 mM ATP; 0.16 mM NAD^+; 3.0 μg of aldolase; and 3.0 μg of glyceraldehyde-3-phosphate dehydrogenase. The final pH of this mixture was 8.4 and provided conditions for maximal enzyme activity.

REACTION MIXTURE B. This solution was used to study activation of the enzyme by cyclic 3′,5′-AMP and related adenylic nucleotides. It was similar to reaction mixture A except that the final concentrations of ATP and FruP were 0.06 and 0.17 mM, respectively, and the pH was adjusted to 6.8.

Controls were included to ensure that the coupling enzyme system was not rate limiting or affected by cyclic 3′,5′-AMP. Fructose bisphosphate ($FruP_2$), the product of the phosphofructokinase reaction, was used as the substrate for the coupling enzyme system. The reduction of NAD^+ by glyceraldehyde-3-phosphate dehydrogenase was directly proportional to the $FruP_2$ concentration tested, indicating that none of the components of the coupling system were rate limiting. No lag in NADH formation was observed. The rate of NADH formation was unaffected by the presence of cyclic 3′,5′-AMP (10^{-4} M). The effect of cyclic 3′,5′-AMP in the initial velocity kinetic studies was therefore attributed to an effect on phosphofructokinase activity. Several experiments reported under Results (Figures 1–4) were repeated using a modified coupling system containing a large excess of the coupling enzymes. The following reagents were contained in a final volume of 1.0 ml: 70 mM Tris buffer, pH 9.0; 10 mM sodium arsenate; 10 mM sodium phosphate, pH 7.4; 3.4 mM cysteine; 3.4 mM magnesium chloride; 1.4 mM NAD^+; 100 μg of aldolase; and 100 μg of glyceraldehyde-3-phosphate dehydrogenase. All other conditions were as described for reaction mixtures A and B. The results obtained with the modified coupling system were identical with those reported under Results.

Cuvets containing the assay system were equilibrated to 37° for approximately 10 min prior to initiation of the reaction by the addition of the substrate FruP. Equilibration of the enzyme and cyclic 3′,5′-AMP in the cuvet for 10 min would ensure complete interaction in the event that the response of the enzyme to effector was slow. Further experiments, however, indicated that addition of cyclic 3′,5′-AMP to a reaction in progress resulted in instantaneous activation. This observation would indicate that the interaction of human erythrocyte phosphofructokinase with cyclic 3′,5′-AMP occurs within seconds in contrast to enzymes from other sources.

Desensitization of Phosphofructokinase. The enzyme was subjected to photooxidation in the presence of Methylene Blue and light according to the procedure of Ahlfors and Mansour (1969). A 0.25-ml enzyme solution containing 2×10^{-6} M Methylene Blue was placed in a 12-ml centrifuge tube immersed in ice water and shaken manually at intervals.

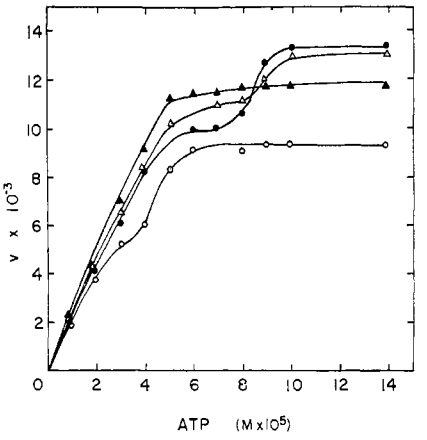

FIGURE 2: Plot of the initial reaction velocity *vs.* the molar concentration of ATP at several fixed FruP concentrations at pH 8.4. ATP concentration was varied between 1×10^{-5} and 1.5×10^{-4} M. Velocities are expressed as nmol of $FruP_2$ formed/10 min per ml of enzyme. (○) 1×10^{-4} M FruP; (●) 4×10^{-4} M FruP; (△) 8×10^{-4} M FruP; (▲) 1.2×10^{-3} M FruP.

Photooxidation was allowed to proceed for approximately 10–15 min, with a 250-W reflector bulb 20 cm above the tube. Control experiments were run in the presence of Methylene Blue, but without exposure to light.

Experimental Results

Preliminary experiments showed that enzyme activity is maximal at a pH of roughly 8.4, and falls at higher and lower values. At pH 6.8, the activity was approximately 18% of the maximum.

Initial Velocity Studies at pH 8.4. When rates were measured at varying FruP concentrations at several fixed concentrations of ATP, a pronounced deviation from a rectangular hyperbola was consistently observed (Figure 1). An intermediary plateau occurs in the substrate concentration–velocity plot at approximately 10^{-4} M FruP. In a double reciprocal plot the data produce biphasic curves, with two apparent K_m and V_{max} values at each concentration of ATP.

When ATP was used as a variable substrate at several fixed concentrations of FruP, marked deviation from Michaelis–Menten behavior was again observed except at the highest FruP concentration, 1.2 mM (Figure 2). Here, V_{max} was reduced, indicating substrate inhibition.

With varying concentrations of $MgCl_2$, at saturating concentrations of ATP (0.35 mM) and FruP (1.4 mM), a simple saturation curve was observed. The K_m value for $MgCl_2$ was ~0.06 mM.

Phosphofructokinase kinetic patterns were investigated at pH 8.4 in the presence of the following nucleotides, all at 10^{-4} M: IMP, IDP, ITP, GMP, GDP, GTP, UMP, UDP, AMP, ADP, and cyclic 3′,5′-AMP. In no case was the behavior altered.

Citrate, which inhibits the enzyme from other sources at concentrations as low as 10^{-5} M, was also tested. There was little loss of activity down to 10^{-2} M citrate, though at very high concentrations enzyme activity progressively decreased until complete inhibition occurred at 0.1 M citrate. The latter effect can be explained by chelation of essential Mg^{2+} rather than addition to the enzyme.

Kinetic Studies at pH 6.8. At this pH, adenine nucleotides have been reported to be activators of the enzyme isolated from other sources. In the present study, AMP and cyclic 3′,5′-AMP activated the enzyme, to a degree that varied with the enzyme preparation. When added simultaneously, the action of these compounds was neither synergistic nor additive. The activation was highest at pH 6.8, declining as the pH was raised.

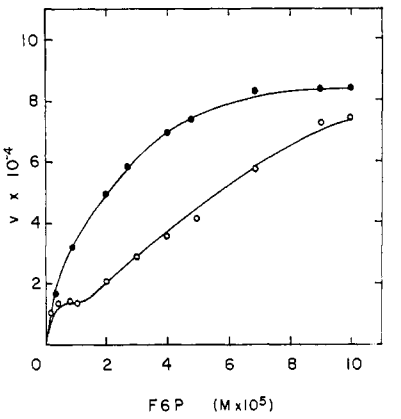

FIGURE 3: Plot of the initial velocity *vs.* FruP concentration at pH 6.8 at an ATP concentration of 0.06 mM. Initial reaction velocities are expressed as nmol of $FruP_2$ formed/10 min per ml of enzyme: (○) control with no cyclic 3′5′-AMP; (●) with 10^{-4} M cyclic 3′,5′-AMP.

The possible effects of cyclic 3′,5′-AMP on kinetic patterns at pH 6.8 were investigated. When the FruP concentration was varied at a fixed concentration of ATP (0.17 mM), deviation from Michaelis-Menten behavior was observed in the absence of added cyclic nucleotide (Figure 3), as at pH 8.4. In the presence of the cyclic nucleotide (10^{-4} M), the biphasic curve became a simple hyperbola. As may be seen from Figure 3, the apparent K_m for FruP was now shifted to a value intermediate between the two K_m values of the original preparation, but V_{max} was not much altered.

When the ATP concentration was varied at a fixed concentration of FruP at pH 6.8, substrate inhibition was seen beyond 0.3 mM (Figure 4). In the presence of cyclic 3′,5′-AMP (10^{-4} M), this inhibition was less severe (Figure 4). The behavior did not depend on the order of addition of ATP and cyclic AMP.

Effects of Photooxidation with Methylene Blue. The time course of loss of enzyme activity during photooxidation treatment was first investigated. A progressive decline in activity was observed, until only 33% remained after 30 min (Table I).

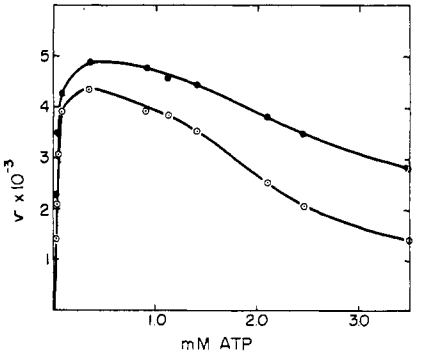

FIGURE 4: Effect of cyclic 3′,5′-AMP on ATP inhibition at pH 6.8. Assay conditions for reaction mixture B were used and are as given in the text. Velocities are expressed as nmol of $FruP_2$ formed/10 min per ml of enzyme: (○) control; (●) with 10^{-4} M cyclic 3′,5′-AMP.

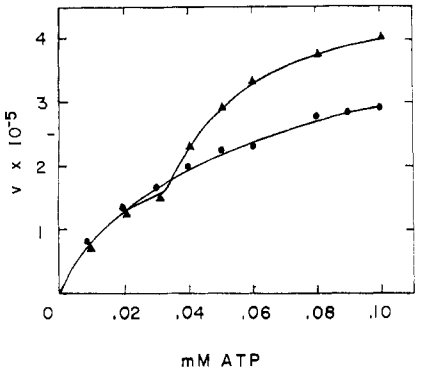

FIGURE 5: The effect of photooxidation treatment of phosphofructokinase on initial velocity kinetic studies with varying ATP concentration and a fixed FruP concentration (10^{-4} M) at pH 8.4. Initial reaction velocities are expressed as nmol of $FruP_2$ formed/10 min per ml of enzyme. Photooxidation was carried out for 12 min in the presence of 10^{-6} M Methylene Blue and light as described under Materials and Methods: (▲) "control" enzyme; (●) photooxidized enzyme.

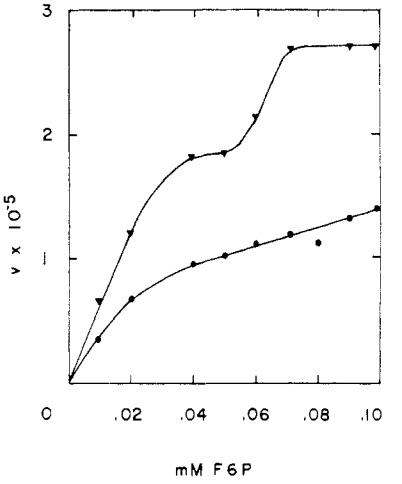

FIGURE 6: The effect of photooxidation treatment of the enzyme on initial velocity kinetic studies with varying FruP concentration and a fixed ATP concentration (8×10^{-5} M). Photooxidation was carried out for 12 min in the presence of Methylene Blue and light: (▲) "control" enzyme; (●) photooxidized enzyme.

A suitable period of treatment for the preparation of desensitized enzyme appeared to be between 10 and 15 min, when the enzyme retained 70–80% of its activity.

With the control enzyme (which had been exposed to Methylene Blue but not light) a biphasic curve was obtained at pH 8.4, as before, when the ATP concentration was varied between 10^{-5} and 10^{-4} M at a fixed concentration of FruP, 10^{-4} M (Figure 5). The two apparent K_m values were 1.4×10^{-5} and 5×10^{-5} M. After photooxidation, Michaelis–Menten kinetics were observed, giving one K_m value of 2.5×10^{-5} M. When the results were plotted according to the Hill equation, the interaction coefficient (n) was 1.06 for the photooxidized enzyme. The control enzyme gave a nonlinear Hill plot with a maximum interaction coefficient (n) of approximately 2.0.

Similarly, when FruP was varied between 10^{-5} and 10^{-4} M and ATP was fixed at 8×10^{-5} M, the control enzyme produced a biphasic curve at pH 8.4 (Figure 6). The apparent K_m values were 1.5×10^{-5} and 6.2×10^{-5} M. This plateau region, although consistently present in repeat experiments, has varied in position with different enzyme preparations (see Figure 1). After photooxidation treatment, Michaelis–Menten kinetics were observed, with one K_m value for FruP of 4.8×10^{-5} M. When plotted according to the Hill equation, the interaction coefficient was 1.0 for the photooxidized enzyme preparation. With the control enzyme the Hill plot was nonlinear, as with varying ATP, and the maximum n value was 4.0.

At pH 6.8, with varying FruP concentrations and at a fixed concentration of ATP, a biphasic curve was observed with the control enzyme (Figure 7). The apparent K_m values for the two sections of the curve were 2×10^{-6} and 5×10^{-5} M. After photooxidation treatment the intermediate plateau region was eliminated. The half-saturation constant for the new curve was roughly 1×10^{-5} M.

Prior photooxidation treatment of the enzyme did not alter the shape of the ATP inhibition curve (see Figure 4). After photooxidation treatment of the enzyme, however, the stimulatory effect of cyclic 3′,5′-AMP on the enzyme was abolished.

Discussion

The most striking feature of the present observations is the existence of intermediary plateau regions in many of the substrate saturation curves, which disappear in the presence of cyclic AMP as well as after photooxidation treatment of the

TABLE I: Time Course of Photooxidation Treatment on Phosphofructokinase Activity.

Time of Photooxidation (min)	Phosphofructokinase Act.[a] × 10^4		
	Control[b]	Photooxidized Phosphofructokinase	% of Control
0	2.55	2.55	100
7	2.55	2.20	86
15	2.55	1.70	67
20	2.55	1.43	56
30	2.55	0.84	33

[a] Expressed as nmol of $FruP_2$ formed/10 min per ml of enzyme. [b] Phosphofructokinase in the presence of 10^{-6} M Methylene Blue but not exposed to light.

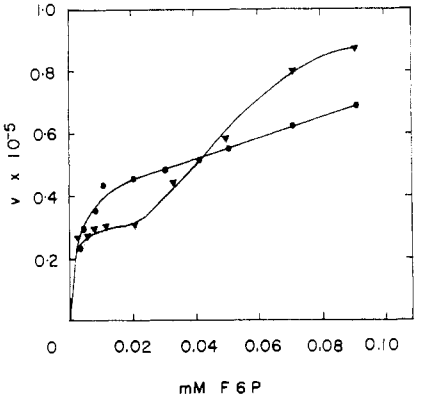

FIGURE 7: The effect of photooxidation treatment of the enzyme on initial velocity kinetic studies at pH 6.8 with FruP as the variable substrate. Photooxidation was carried out for 10 min in the presence of Methylene Blue and light: (▲) "control" enzyme; (●) photooxidized enzyme.

enzyme. These plateau regions are also dependent upon the pH. In the case of ATP saturation curves, a plateau was seen at pH 8.4 but not 6.8, and with FruP the position of the plateau in the curve differed at the two pH values.

A further point of interest is that activation by cyclic AMP occurs at pH 6.8 but not 8.4, and is abolished by the photooxidation treatment. In the light of these observations, the plateau regions and cyclic AMP activation are likely to depend on closely linked mechanisms. On the other hand, the substrate inhibition seen with ATP is probably an independent phenomenon, since it persists after photooxidation. The somewhat diminished substrate inhibition in the presence of cyclic AMP (Figure 6) could, by itself, suggest competition between the latter and ATP for the same site, causing activation when cyclic AMP is bound and inhibition when ATP is bound. Judging by the other observations, however, two different sites are probably involved, which are subject to allosteric interaction.

Other studies of human erythrocyte phosphofructokinase have been described by Layzer *et al.* (1969) and Staal *et al.* (1972). In neither case were intermediate plateau regions seen at pH 8 with FruP or ATP. The cause of this discrepancy could be in differences in the enzyme purification procedure or assay conditions. That our observations are indeed meaningful is attested by the abolition of the plateaus either after photooxidation of the enzyme or in the presence of cyclic AMP. These observations, especially the former, indicate that the complex behavior arises from the properties of the enzyme itself, rather than from experimental artifacts.

The significance of intermediary plateau regions in enzyme saturation curves was explored by Teipel and Koshland (1969), who concluded that such behavior can only occur when two conditions are met. First, the enzyme must possess more than two substrate binding sites. Second, the relative magnitude of the intrinsic catalytic or binding constants of these sites must first decrease and then increase as the enzyme is saturated. The initial decrease is a form of behavior designated in an earlier paper as negative cooperativity (Levitzki and Koshland, 1969), and the increase is an example of the familiar "positive cooperativity" observed long ago with hemoglobin and since extended to many other systems. These conditions may be shown, however, not to be unique determinants of plateau phenomena. Theoretically, indistinguishable behavior could be produced by two independent sites of differing affinity: that with higher affinity is assumed to be subject to substrate inhibition (which can be accounted for by a single binding region, the catalytic site, as in the case of acetylcholinesterase, Krupka and Laidler (1961); and both sites are assumed to exhibit normal Michaelis–Menten kinetics in other respects. Alternatively, the combination of high affinity sites with Michaelis–Menten kinetics and two or more sites of lower affinity showing positive cooperativity could give curves with an almost flat middle region. The unambiguous interpretation of such data therefore requires information ruling out these possibilities.

There is reason to think, however, that a mechanism of the kind envisaged by Teipel and Koshland (1969) must be at work here, involving positive if not negative cooperativity, rather than independent sites. In removing the plateau, photooxidation treatment shifts the apparent K_m to a value intermediate between the two K_m values exhibited by the original preparation. Photooxidation could through chance destroy one of two independent sites, that with higher affinity, but in order to account for the observations it would also have to raise the affinity of the other site for both substrates, without much altering its catalytic activity. While this is not impossible, such a combination of events is most unlikely. Photooxidation may therefore produce changes in protein structure which lead to an altered pattern of attractive forces between subunits and greatly attenuated allosteric interactions. As a result, a modifier such as cyclic AMP bound at a regulatory site, or a substrate at a catalytic site, would fail to influence catalysis at other centers.

If the observed behavior is indeed due to positive and negative cooperativity, then two different types of mechanism could conceivably operate. One involves subunit interactions in a multisubunit enzyme containing several catalytic and regulatory sites. The other involves several forms of the enzyme of different aggregate size, the reversible association of subunits being influenced by the concentrations of substrates and modifiers. As noted in the introductory statement, evidence for both reversible association and polymeric forms of differing enzymic activity was obtained earlier with heart phosphofructokinase.

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Dependency on Environmental Redox Potential of Photophosphorylation in *Rhodopseudomonas spheroides*†

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ABSTRACT: An all-glass apparatus was constructed which allowed measurement of photophosphorylation at different redox potentials. The luciferin–luciferase assay was adapted so that ATP could be measured within a few minutes of its formation. Optimal photophosphorylation activity in chromatophores from *Rhodopseudomonas spheroides* under saturating white light was 150 μmol of ATP/hr per mg of bacteriochlorophyll and was found at an environmental redox potential near 0 V at pH 7.9. Activity decreased at lower potentials, with a midpoint for the transition occurring at −0.09 V at pH 7.9 and −0.03 V at pH 7.0. The latter value is close to that for decreased phototrap activity measured previously by following light-induced absorbance changes (E_0' = −0.02 V). Photophosphorylation activity also decreased at higher potential with an apparent midpoint at 0.30 V. This is the first measurement of such a redox-linked component in photophosphorylation.

The primary events in bacterial photosynthesis have been shown to occur only within a limited range of environmental redox potential (Loach *et al.*, 1963; Kuntz *et al.*, 1964; Loach, 1966; Cusanovich and Kamen, 1968a,b; Cramer, 1969; Nicolson and Clayton, 1969; Reed *et al.*, 1969; Dutton, 1971; Case and Parson, 1971; Dutton and Jackson, 1972). Thus, light energy is ineffective in driving normal light-dependent reactions at environmental potentials above +0.4 V because the primary electron donor becomes oxidized (E_0' = +0.44 V for *Rhodopseudomonas spheroides*). These systems are also unable to utilize absorbed light energy when the environmental potential is below 0 V (E_0' = −0.02 V for *R. spheroides*); it is now generally assumed (Nicolson and Clayton, 1969; Reed *et al.*, 1969; Dutton, 1971; Leigh and Dutton, 1972; Dutton and Jackson, 1972; Jackson *et al.*, 1973; Dutton and Leigh, 1973) that the reason for this is because the primary electron acceptor molecule becomes reduced at lower potential, although other explanations should also be given serious consideration (Loach and Katz, 1973; Loach, 1973). What does seem clear is that the primary photochemistry is attenuated with a midpoint potential of −0.02 V for *R. spheroides* chromatophores. Considering these limitations on the primary photochemical event, all subsequent reactions such as electron transport, photophosphorylation, and energy-dependent ion transport should also be restricted to operating within the same redox potential range.

Earlier studies on photophosphorylation in chromatophores prepared from *R. rubrum* showed that photophosphorylation is sensitive to reductants and that there is no activity under highly reducing conditions (Frenkel, 1956; Newton and Kamen, 1957; Geller, 1957; Vernon and Ash, 1960; Horio and Kamen, 1962; Bose and Gest, 1963; Gest, 1963; Cusanovich and Kamen, 1968b). It was also clear from these studies that the photophosphorylation activity decreased under oxidizing conditions so that there seemed to be a somewhat narrow ideal range of redox potential in which the system was active. Among the effects reported upon adding various chemicals to the photophosphorylating system, an electron donor system seemed to be required (Frenkel, 1956) for the best activity (*e.g.*, succinate, ascorbate). Previous studies (Loach *et al.*, 1963; Loach, 1966) had indicated that intact photosynthetic bacteria seem to be substantially redox buffered and have an environmental potential near 0 V. These observations suggested to us that photophosphorylation activity might be highly dependent on the redox potential of the environment and that such a dependency might be used to turn the energy yielding system on and off as needed. Bose and Gest (1963) have made a similar suggestion.

The controlled redox potential method of measuring the dependency of a biological activity on linked redox transitions in preparations of complex membranous fractions derived from whole cells was first employed by Kok (1961) to determine the E_0' value of the primary electron donor of system I in an acetone-extracted chloroplast system. General development of the method for studying *in vivo* systems over extended ranges of redox potential and the adoption to anaerobic conditions were carried out by Loach (Loach *et al.*, 1963; Kuntz *et al.*, 1964; Loach, 1966). More recently, the latter techniques have been applied to a variety of complex systems where evidence for a redox-linked component has been obtained even though no other data were known which gave properties of the component. We would like to report

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