*A CHANGE IN OPTICAL ROTATION OF CREATINE-ATP
TRANSPHOSPHORYLASE DURING ENZYME SUBSTRATE
INTERACTION SUGGESTING AN ALTERATION IN CONFORMATION**

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Communicated by Walter H. Stockmayer, October 16, 1961

One of the main tasks in the study of enzyme function is the elucidation of evidence for mechanisms which express the complex, highly organized structures of proteins. The deformation of enzyme in response to substrate has been recently thoroughly discussed as a possible contributory mechanism to enzyme action,¹ but evidence reported has been primarily thermodynamic and kinetic. This paper presents evidence based on a decrease in optical rotation, observed when the enzyme is in equilibrium with its substrates, which when combined with recently published immunoenzymological experiments on creatine kinase strongly suggests that a conformation change occurs.²

At the time creatine-ATP-transphosphorylase (Cr-kinase) was crystallized, preliminary studies were carried out which showed that this enzyme was more completely protected against thermal denaturation by the simultaneous presence of all of its substrates (MgATP-Cr) than by any one of them singly.^{3a} More recently, while working with antibodies to myosin and 5'adenylic acid deaminase,^{4a} which have been shown to change configuration (measured immunochemically) during denervation atrophy,^{4b} it was shown that although the substrate 5'adenylic acid protected the deaminase against antibody inhibition, a competitive inhibitor, inorganic phosphate, increased the affinity of the enzyme to its specific antibody.² Furthermore, while working with the Cr-kinase antibody system one of us (A.J.S.) found that although each of the substrates was independently bound to the enzyme, MgATP or Mg plus Cr did *not* "protect" this enzyme against antibody inhibition, while the simultaneous addition of MgATP and Cr *did* successfully deter antibody inhibition, although the equilibrium constant of antibody-enzyme combination is approximately a millionfold that of the enzyme-substrate affinity.² These data were interpreted as indicating that there was a change in conformation of these enzymes during substrate combination,² and led to the present experiments.

In order to obtain further evidence suggesting a change in conformation, a study of the optical rotation of Cr-kinase was undertaken. Cr-kinase is an ideal enzyme with which to study the effects of substrate binding, for at equilibrium with its substrates it is primarily in the bound form. The over-all equilibrium constant (Cr) (MgATP)/(Cr-P)(MgADP) is of the order of unity.^{3b}

The Michaelis constants for the substrates in both forward and reverse direction were measured, and it was shown that the Michaelis constant is equivalent to the equilibrium constant of the dissociation reaction of the enzyme and a given substrate;^{5a} i.e., the values for MgATP, MgADP, Cr, and Cr-P are 0.40, 0.2, 19.0, and 0.8 mM respectively.^{5b}

Materials and Methods.—Six different preparations of once- and twice-crystallized creatine kinase were used, at concentrations that varied from 0.4 to 1.6 per cent,

usually at pH 9.0 in 0.1 *M* glycine buffer (but some experiments were run at 7.0). These were fully active preparations containing approximately 70 units/mg activity. The protein concentration was measured by Biuret method. Checks on enzyme concentration after removal from polarimeter were also run.

Optical rotation was measured by means of a model 80 photoelectric spectropolarimeter with an oscillating polarizer prism (O. C. Rudolph and Sons) at a wavelength of 366 $m\mu$ with a mercury lamp. The rotatory dispersion curves were run between 313 and 589 $m\mu$.

Results.—Figure 1 shows the rotatory dispersion of the enzyme alone, in the

Fig. 1.—Rotatory dispersion of creatine kinase at pH 9.0 in glycine (0.1 *M*). Substrate concentrations at equilibrium Mg^{++} = 20.0 mM; ATP = 1.3 mM; ADP = 2.6 mM; Cr = 21.2 mM; Cr-P = 2.4 mM.

presence of all substrates, and in 6 *M* urea. The equilibrium concentrations of substrates calculated from earlier data^{3b} may be seen in the legend. The “working” enzyme in combination with its substrates shows a small but consistent increase in the specific rotation in the positive direction. Urea increases negative rotation. All three curves give excellent straight lines when plotted according to the single term Drude equation, as do most globular proteins. Creatine kinase fits into the first group of Jirgensons’ classification.⁶ The λ_c (normally 252 $m\mu$) as may be seen from Table 1, part 5, changes extensively on urea denaturation (220 $m\mu$). The small change in this value seen on substrate combination (252 to 258 $m\mu$) is probably not significant. The α_D^{25} of this protein is approximately 38 and decreases to 34 when the substrate is bound. Experiments have also shown that the specific rotation increases linearly in proportion to the amount of substrate bound. The small change in specific rotation is very close to those values found with D-glyceraldehyde-3-P dehydrogenase,⁷ when this enzyme was crystallized in the presence or absence of

TABLE 1
CHANGE OF OPTICAL ROTATION OF CREATINE KINASE ON BINDING SUBSTRATES

Sample		$-(\alpha)_{266}^{23}$	Remarks
1.	E	178 (-2.523)	(Protein) = 1.41 gm%
	E + S	162 (-2.284)	S = Cr + ATP + Mg which rotates more negative on forming CrP + ADP
	S	... (-0.04)	
2.	E + ATP + Mg	172	(Protein) = 1.45 gm%
	E + ATP + Mg + Cr	163	Cr - no rotation CrP negative slope as concentration increases; ADP more negative than ATP
3a.	E + S + EDTA	171	EDTA (0.017 M) added after equilibration of E + S (Protein) = 0.73 gm%
	E + S	165	
b.	E + S + EDTA	172	EDTA (0.033 M) as above $(\alpha)_D^{23}$ 37.5
	E + S	160	(Protein) = 1.13 gm% 34.0
4.	E	168	pH 7.0; Enzyme 60% bound; change in rotation proportional to binding; (Protein) = 0.4 gm%. Rotation changed by competitive inhibitor
	E + S	160	
		λ_c	K
5.	E -	252 m μ	132×10^5
	E + S	258 m μ	106×10^5
	E + Urea	220 m μ	292×10^5

DPN. Elodi and Szabolcsi⁸ obtained even greater differences with the same enzyme when it was crystallized in the presence or absence of DPN. Table 1 further shows that the specific rotation of creatine kinase undergoes a change only in the presence of all substrates (when it is "working"), rather than when only two of the substrates are added (see part 2 of Table 1). This experiment is reminiscent of the immunoenzymological experiments² on which it was patterned. Of interest is the fact that although MgATP and creatine are separately bound⁹ any new asymmetric center that might be expected to be formed especially by the MgATP-protein does not appear to change the optical rotation. The rotatory contribution of the substrates is very small when compared with that of the protein and in the opposite direction to the observed effect.† This, however, does not completely rule out the possibility that a new asymmetric center is formed.¹⁰ In order to test whether the equilibrium mixture of the substrates contributed to the rotation, ADP and Cr-P were also tested. The ADP had a more negative rotation than the ATP, and the Cr-P had negligible rotation at the concentrations used; but when higher concentrations were used the rotation became more negative rather than positive as was observed when the protein was bound. Ethylene diamine-tetraacetate (EDTA) has been shown to inhibit the enzyme when added in amounts stoichiometric with the Mg in the enzyme system.^{11a} When EDTA was added to the enzyme-substrate equilibrated mixture, the rotation was shown to increase to the unbound form. Controls at several different levels of EDTA, protein, and substrates combined and uncombined were also run, and this result obtains at all levels high enough to bind the Mg⁺⁺. The pH dependence of the rotation has been found to vary only slightly between pH 5 and pH 9; a very slight minimum (much less than the differences observed with substrate combination) exists at pH 7.5. Beyond these limits the rotation becomes more negative. Other experiments which were done at pH 7.0, to rule out any possible pH effect, showed the same changes as at pH 9.0 (see part 4, Table 1).

The effect of paramercuribenzoate (PMB) on the specific rotation of the enzymes was tested and shown to be negligible, although this sulfhydryl reagent binds to the protein and modifies the K_m .^{11b}

NEM (N-ethyl-maleimide), which also modifies K_m and V_{max} , causes a positive change in rotation comparable to that of substrate when two molecules of the inhibitor are bound.

Discussion.—Although the role of conformation changes in enzyme mechanism has been thoroughly discussed and the “detection of small configurational changes accompanying substrate binding” described as of considerable importance,¹² the present results are the first among the undoubtedly large number of enzymes that have been studied by optical rotation that have shown a specific change during normal substrate activation. The fortuitous relationship of high binding constants of the substrates, and the over-all equilibrium close to unity in the ratio of the substrates of the forward and reverse reaction are probably responsible for this finding.

The very recent results of Szabolcsi and Biszku¹³ on aldolase showing that PMB caused changes in conformation as tested by trypsin digestion, which were reversible by substrate addition, prior to complete enzyme inactivation, may be very similar to the effect of NEM, but not PMB, described here.

The present optical rotation results on creatine kinase support the earlier immunoenzymological results² pointing up the value of the biological tool that has, until recently, been neglected except by a few.^{4, 14}

These results strongly suggest on the basis of physical criteria that a conformation change is important for enzyme action and suggest further experiments to determine whether the process involved in this conformation change is one of “induced fit” as proposed by Koshland¹ or whether there is an exchange of energy and information between the substrate and the enzyme which somehow triggers a response in the protein coils.

Summary.—The specific rotation and rotatory dispersion of creatine-ATP-transphosphorylase has been measured and found to follow the single term Drude equation between 313 and 589 $m\mu$. The enzyme has been found to undergo a small but consistent increase in specific rotation only when in equilibrium with *all* of its substrates, which suggests a conformation change during activity.

Addendum.—Although the dispersion data shown in Figure 1 give good lines when plotted according to the Yang-Doty method, it was decided, after a conversation with Dr. A. G. Szent-Györgyi about this work, to attempt a Moffitt plot of these data in order to obtain b_0 value, which is often a measure of the helical content.¹⁵ $\alpha^{23}(\lambda^2 - \lambda_0^2) \times 10^{-6}$ versus $1/(\lambda^2 - \lambda_0^2) \times 10^6$ was used, assuming a λ_0 of 210 $m\mu$. The native and bound enzyme gave fairly good straight lines although the difference between the b_0 values (254 and 215) was within the experimental error of the data. The urea-treated enzyme gave a very poor line (b_0 anywhere from -33 to -111). This work is being continued.

The authors are indebted to Drs. P. von Hippel and W. Englander for discussions concerning optical rotation and to K. Y. Wong for the extremely careful help he rendered in operating the spectropolarimeter.

* This work was presented by Arthur Samuels at the Biophysical Society Meeting 5 TB-9, 1961, and was supported by a grant from the National Science Foundation (13083).

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‡ This may be seen from the values in parentheses in Table 1, part 1.

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PHOSPHATE INCORPORATION INTO ALKALINE PHOSPHATASE OF *E. COLI**

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Communicated October 19, 1961

Serine phosphate has interested us for a long time.^{1, 2} More recently, the function of serine as a component of an active center in enzymes has received much attention.^{3, 4} The quality of the hydroxyl group in serine is obviously variously modified by neighboring amino acids and, more generally, by structural features of the molecular environment.^{5, 6} Now, the reports of Ågren,⁷ and of Engström⁸ on the isolation of serine phosphate from phosphate-incubated mammalian alkaline phosphatase make it seem likely that serine is the active center of this enzyme. The availability of the phosphatase of *E. coli* in a rather pure form⁹ made us hopeful that we might here have an opportunity to obtain precise information on the situation of the reactive serine in this enzyme. This seemed particularly desirable since the genetic characterization of the phosphatase is far advanced.¹⁰⁻¹² We hoped eventually to map the amino acid sequence at the active center by marking serine in the enzyme with radioactive phosphate. In accord with observations on other phosphatases, we found that after incubation with inorganic phosphate, serine phosphate can be isolated from *E. coli* phosphatase. Similar results with this enzyme were obtained independently by Engström.¹³