now available should provide a much needed means to study the importance of this residue in these systems.

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Effect of Ligands on the Reactivity of Essential Sulfhydryls in Brain Hexokinase. Possible Interaction between Substrate Binding Sites†

Vilas D. Redkar and Umakant W. Kenkare*

ABSTRACT: Inactivation of bovine brain mitochondrial hexokinase by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), a sulfhydryl specific reagent, has been investigated. The study shows that the inactivation of the enzyme by DTNB proceeds by way of prior binding of the reagent to the enzyme and involves the reaction of 1 mol of DTNB with a mol of enzyme. At stoichiometric levels of DTNB, the inactivation of the enzyme is accompanied by the formation of a disulfide bond. But it is not clear whether the disulfide bond or the mixed disulfide intermediate formed prior to it causes inactivation. On the basis of considerable protection afforded by glucose against this inactivation it is tentatively concluded that the sulfhydryl residues involved in this inactivation are at the glucose binding site of the enzyme, although other possibilities are not ruled out. An analysis of the effects of various substrates and inhibitors on the kinetics of inactivation and sulfhydryl modification by DTNB has led to the proposal that the binding of substrates to the enzyme is interdependent and that glucose and glucose 6-phosphate produce slow conformational changes in the enzyme. Protective effects by ligands have been employed to calculate their dissociation constants with respect to the enzyme. The data also indicate that glucose 6-phosphate and inorganic phosphate share the same locus on the enzyme as the γ phosphate of ATP and that nucleotides ATP and ADP bind to the enzyme in the absence of Mg^{2+}.

A recent communication from our laboratory (Redkar and Kenkare, 1972) briefly described experiments bearing on the role of sulfhydryl residues in the activity of brain hexokinase. Using DTNB,[1] a thiol specific reagent, evidence was presented to indicate that one or two sulfhydryl

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[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid), Glc-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; TNB, thionitrobenzoate anion.

residues were crucial for the activity of this enzyme. On the basis of protective effects by glucose and Glc-6-P, it was suggested that these thiol groups might be present at the enzymic active site. Studies on the effect of tetranitromethane on the activity of brain hexokinase have provided additional support to this suggestion (Subbarao et al., 1973). In this report we present data to show that the inactivation of the enzyme by DTNB proceeds by way of prior binding of the reagent to the enzyme and involves the reaction of 1 mol of DTNB with a mol of enzyme. On the basis of experiments using low molar excess of DTNB over the enzyme, we further propose that this inactivation is accompanied by the formation of a disulfide bond. A study of the influence of various ligands, on the reaction between DTNB and hexokinase, also leads us to the conclusion that there is interaction between the various ligand binding sites of the enzyme.

Materials and Methods

Chemicals. Glucose-6-phosphate dehydrogenase, lactic dehydrogenase, pyruvate kinase, $NADP^+$, NADH, ATP, and Glc-6-P were obtained from Boehringer, Mannheim, West Germany. 2-Mercaptoethanol was a product of Koch-Light Laboratories, Colnbrook, Bucks, England. DTNB was supplied by Pierce Chemical Company. Analar grade EDTA was a product of BDH division of Glaxo Laboratories, Bombay, India. Crystalline bovine albumin used as a standard for measurement of protein concentration was obtained from Nutritional Biochemicals Corporation. Bovine brain mitochondrial hexokinase was prepared by a previously published procedure (Redkar and Kenkare, 1972).

Enzyme Assays. Hexokinase was assayed either by coupling the formation of Glc-6-P to the reduction of triphosphopyridine nucleotide (Sharma et al., 1963) as described previously (Redkar and Kenkare, 1972), or by coupling the formation of ADP to the oxidation of NADH as described by Grossbard and Schimke (1966). Unit of activity has been defined earlier (Redkar and Kenkare, 1972).

Estimation of Sulfhydryl Residues. These were estimated by the method of Ellman (1959) using DTNB as described in detail by Redkar and Kenkare (1972). For these experiments, the stock solution of the enzyme (Redkar and Kenkare, 1972) was dialyzed against 0.05 *M* Tris-chloride buffer (pH 8.0) containing 0.5 m*M* EDTA. Experiments involving DTNB were carried out at 25°.

Protein Concentration. Protein concentration was determined by the method of Lowry et al. (1951).

Results

Effect of Various Metabolites on the Reactivity of Sulfhydryl Residues of the Enzyme. Sulfhydryl residues in bovine brain hexokinase have differing degrees of reactivity toward DTNB (Redkar and Kenkare, 1972). These reactivities are modified differently by various metabolites related to hexokinase. The protective effects of these ligands against inactivation by DTNB also vary to a considerable extent. We described these effects in qualitative terms in our previous communication (Redkar and Kenkare, 1972). To facilitate the interpretation of experiments reported in this paper, it is necessary to describe here in greater detail the results which were briefly referred to earlier.

Figure 1 describes the progress of reaction of sulfhydryl residues of the enzyme with excess DTNB. The curved line represents the composite modification of about 11 out of 12 free sulfhydryl residues of the enzyme by excess reagent. It can be seen from this figure that we have attempted to resolve and classify the sulfhydryl residues on the basis of their reactivity. As pointed out by Ray and Koshland (1961), the resolution of such a complex curve is not too reliable and for this reason we do not consider this classification as rigorous. But we have found it useful for comparing the effects of various ligands on the reactivity of sulfhydryls and for drawing some tentative conclusions about the binding sites of these ligands.

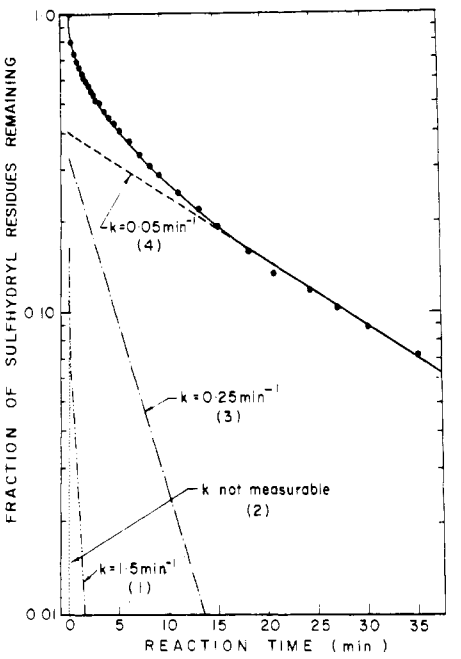

FIGURE 1: Reaction of sulfhydryl residues of hexokinase with DTNB and identification of sulfhydryl residues with different reactivities. Reaction of sulfhydryl residues with DTNB was followed as described under Materials and Methods. The kinetics was followed in spectrophotometric cuvets of 1-cm path length. Both the reaction and reference cuvets contained 104 m*M* Tris-chloride buffer (pH 8.0) and 0.5 m*M* EDTA. The reaction cuvet contained in addition 4.66 μM enzyme. The reaction was started by the addition of 0.833 m*M* DTNB to both reaction and reference cuvet. Since the concentration of DTNB in this experiment was about 180-fold that of the enzyme, first-order conditions may be assumed to have been maintained during the course of the experiment. The graphical analysis of the composite curve shown above (—), for resolving the residues on the basis of their reactivities, is based on the method of Ray and Koshland (1961). The values of k, the first-order rate constant, for different types of sulfhydryl residues have been indicated. The figures in parentheses show the number of residues in each category, rounded to the nearest integer.

Using the same procedure as outlined in Figure 1, the reactivities of sulfhydryl residues were also determined in presence of various metabolites at a fully saturating concentration of 10 m*M*. Table I summarizes the results obtained in presence and absence of these additions. In this table, the sulfhydryl residues have been classified into four broad groups A–D in decreasing order of their reactivity as measured by the pseudo-first-order rate constants of their reaction with DTNB. In view of the uncertainties inherent in this kind of experiment, attention should be directed only to the differential effects of various ligands. The results are briefly analyzed below.

No Ligand Present. Some 10–11 residues out of a total of 12 were found to react with DTNB in the absence of any ligand. Of these, two react too fast for the rate constant of the reaction to be accurately determined (group A). Next in reactivity come residues in group B and these are worthy of attention. They react with DTNB with a rate constant of about 1.0–1.5 min^{-1}. Since the rate constant of inactivation

Table I: Effect of Various Ligands on the Reactivity of Sulfhydryl Residues of Brain Hexokinase toward DTNB.[a]

Ligand Added	Number[b] of Sulfhydryl Residues Classified on the Basis of Pseudo-First-Order Rate Constant of Reaction with DTNB: A (Rate constant too high to be measured)	B (1.0–1.5)[c]	C (0.2–0.5)[c]	D (0.03–0.07)[c]	Total Number[d] of Residues Reacted	Rate Constant of Inactivation[e] in Presence of Ligand
Nil	2.0	1.5	3.0	4.0	10.5	1.5 ± 0.4
Glucose	2.0			7.0	9.0	0.06 ± 0.03
ATP; Mg-ATP	2.0	1.5	2.5	3.0	9.0	1.2 ± 0.2
ADP; Mg-ADP	2.0	1.5	2.5	3.0	9.0	1.5 ± 0.3
P_i	2.0	1.5	2.0	3.5	9.0	1.3 ± 0.1
Glc-6-P	2.0		1.0	1.0	4.0	Too low to be measured
Fru-6-P	2.0		1.0	6.0	9.0	0.06 ± 0.03
Glucose + ATP	2.0			1.5	3.5	Too low to be measured

[a] The procedure for calculating the rate constants and the number of residues possessing the same reactivity has been described in the legend to Figure 1. All ligands were added at 10 m*M* concentration. For other details see legend to Figure 1. [b] The number of residues is the average of five experiments, rounded to the nearest multiple of 0.5 residue. [c] Numbers in parentheses indicate limits within which values for pseudo-first-order rate constants expressed in min^{-1}, were found to vary over a course of several experiments. [d] Number obtained by adding the figures under columns A–D. [e] Rate constant of inactivation expressed in min^{-1} showing the limits of variation over a course of several experiments. The rate constants were determined by following the kinetics of activity loss after addition of DTNB.

of the enzyme by DTNB in the absence of any ligand is also about 1.5 min^{-1} (Table I, last column), it is reasonable to conclude that these residues of category B are important for enzyme activity. The likelihood that other residues with lower reactivity might also be involved in enzyme function is, however, not ruled out.

Glucose. Glucose abolishes the reactivity of sulfhydryl residues in category B toward DTNB and also protects the enzyme substantially against inactivation by this reagent. This result appears to support our above suggestion about the importance of group B residues for the activity of the enzyme. However, it should be noted that glucose also abolishes or modifies the reactivity of sulfhydryl residues in category C and thus may be protecting the enzyme against inactivation by inducing a conformational change in it. The fast reacting residues of group A do not appear to have any role in enzyme function, since they remain unaffected in presence of glucose.

ATP, Mg-ATP, ADP, Mg-ADP, and P_i. It is interesting to note that all these ligands have similar effects on the reactivity of sulfhydryl residues. None of them affect the fast reacting sulfhydryls of group A nor the group B residues. Since none of these ligands protect the enzyme against inactivation by DTNB (Redkar and Kenkare, 1972), the importance of group B residues for the activity of the enzyme is further emphasized. All of these ligands modify the reactivity of residues in group C and group D though to slightly different extents. The protective effect of phosphate is felt mostly by a type C residue and partially by a type D residue. The reverse is the case with ATP and ADP or their complexes with Mg^{2+}. The implication is that these ligands occupy overlapping sites on the enzyme with ATP and ADP sharing the same locus on the enzyme. Another interesting point which emerges is that the protective effects of ATP and ADP on particular sulfhydryls are similar to the effects of their complexes with Mg^{2+}. Since the enzyme gave only a trace of activity in the absence of added Mg^{2+}, it is safe to assume that the enzyme was free from any bound Mg^{2+}.

Glc-6-P. In the presence of this product inhibitor, the high reactivity of sulfhydryls of group A is unaffected, but the reactivity of other sulfhydryls of groups B, C, and D is drastically reduced or abolished. Glc-6-P also protects the enzyme completely against inactivation by DTNB. A small molecule like Glc-6-P cannot change the reactivity of so many sulfhydryl residues by steric hindrance alone. Also if the protective effect of Glc-6-P is due to overlap at more than one site, then it should be about equal to the sum of the separate effects of say glucose and P_i on the enzyme. This is not the case. The result is therefore strongly suggestive of a conformational change produced in this enzyme by Glc-6-P. Such a conformational change has in fact been demonstrated (Redkar and Kenkare, 1972, Chakrabarti and Kenkare, 1974; Wilson, 1973).

Fru-6-P. This ligand which is a product but not an inhibitor of the brain hexokinase reaction (Crane and Sols, 1954) showed an effect similar to that of glucose both as regards protection of sulfhydryls and prevention of activity loss (Redkar and Kenkare, 1972).

Glucose Plus ATP. In the simultaneous presence of these two substrates, the reactivity of sulfhydryls toward DTNB closely resembles that in the presence of Glc-6-P. Also the enzyme is almost fully protected against inactivation by DTNB. The possibility that this effect could have been due to the formation of Glc-6-P in the reaction mixture is, however, ruled out. Only a trace amount of Glc-6-P is formed in this system because of the absence of Mg^{2+} and this trace amount is inadequate to explain the level of protection obtained under the conditions of the experiment. The striking effect of glucose and ATP when added together to the enzyme compared to their effects when added to the enzyme separately deserves attention and will be discussed below in connection with other experiments.

Mechanism of Inactivation of the Enzyme by DTNB. The inactivation of brain hexokinase by excess DTNB at a number of inhibitor concentrations was found to follow pseudo-first-order kinetics for most of the inactivation process. However, increase in the concentration of DTNB did not result in proportionate increase in the first-order rate constant of the inactivation reaction. This indicated saturation of the enzyme by DTNB with a limiting rate constant

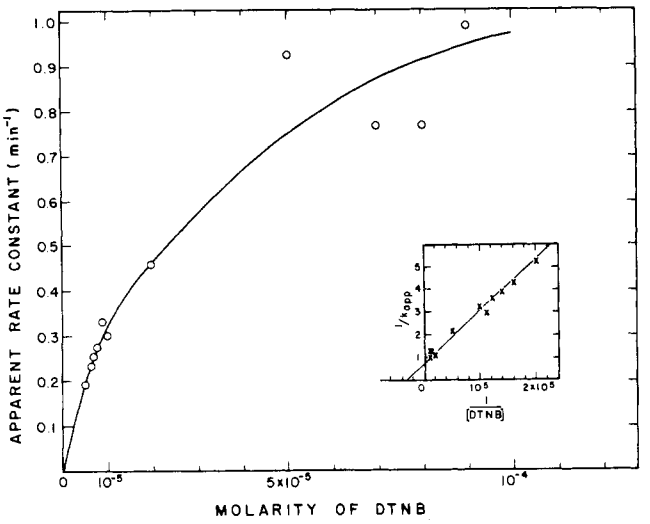

FIGURE 2: Apparent first-order rate constant of inactivation as a function of DTNB concentration. 1 μM hexokinase was treated with various concentrations of DTNB as indicated in the figure. Apparent first-order rate constants of inactivation were determined by following the kinetics of activity loss after addition of DTNB. Inset shows a double reciprocal plot of DTNB concentration vs. rate constant of inactivation.

of inactivation of about 1.0 min^{-1} (Figure 2). The reaction of DTNB with brain hexokinase thus appeared to proceed on the analogy of an enzyme–substrate interaction, through the formation of a reversible complex which broke down irreversibly to yield the modified inactive enzyme and the TNB anions (see below). The inactivation reaction can thus be described as

$$\text{enzyme} + \text{DTNB} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{complex}}{\text{enzyme-DTNB}} \xrightarrow{k_2} \text{modified inactive enzyme} + 2\text{TNB anions} \quad (1)$$

where k_1 and k_{-1} are rate constants of formation and breakdown of enzyme-DTNB complex and k_2 is the pseudo-first-order rate constant of inactivation of enzyme-DTNB complex to give the modified inactive enzyme.

By a simple derivation (Kitz and Wilson, 1962; Pétra, 1971) one can get a Lineweaver-Burk type of relationship between apparent first-order rate constant and concentration of DTNB. A reciprocal Lineweaver-Burk plot of apparent first-order rate constant against concentration of DTNB (inset, Figure 2) gave the kinetic constants of the hexokinase-DTNB complex. The plot gave a limiting rate constant of inactivation k_2 of 1.4 min^{-1}. The dissociation constant K for the enzyme-DTNB complex as calculated from this plot (assuming that in eq 1, k_2 is small relative to k_{-1}) was found to be 3.6×10^{-5} M. It must be noted that the inactivation reaction we have been discussing so far is the one that occurs in the absence of any ligand. The inactivation of the enzyme by DTNB in presence of 10 mM ATP or in presence of 10 mM glucose was also found to proceed through the formation of the enzyme-DTNB complex (results not shown). The dissociation constant for this complex in presence of 10 mM ATP, $K_{(\text{DTNB,ATP})}$, was also found to be 3.6×10^{-5} M. The dissociation constant for the complex in presence of 10 mM glucose, $K_{(\text{DTNB,glucose})}$, was calculated to be 10^{-4} M.

Number of Sulfhydryl Residues Involved in the Inactivation of the Enzyme. Figure 3 describes an experiment in which the enzyme was treated with excess DTNB and the loss in its activity was followed with respect to the sulfhydryl residues modified. It is clear from this figure that the modification of the first two residues has little effect on enzyme activity, but modification of the third and fourth sulfhydryl residues destroys it precipitously. Comparison of these data with that presented in Figure 1 and Table I makes it evident that the first two residues are the fast reacting ones in group A (Table I) and residues 3 and 4 are group B residues considered as important for the activity of the enzyme.

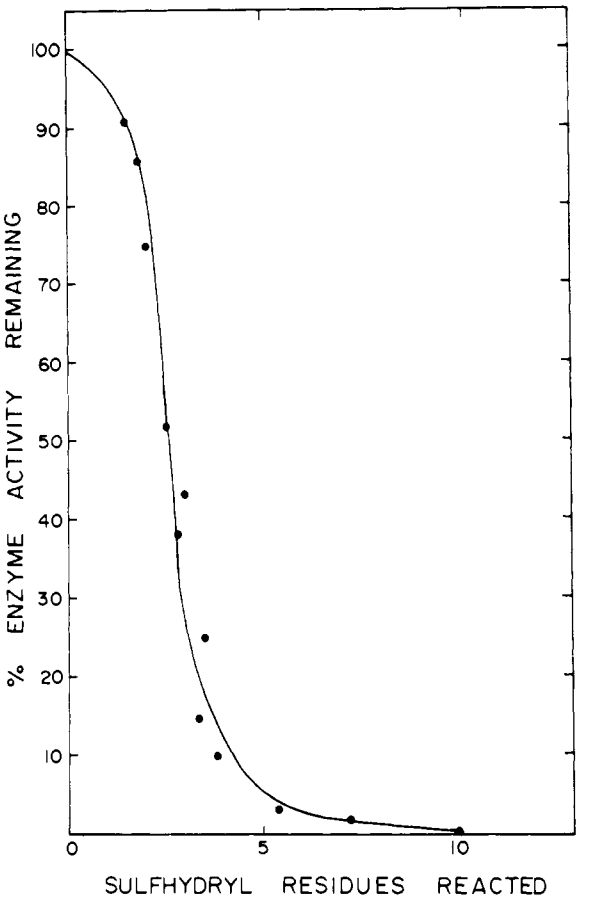

FIGURE 3: Enzyme activity as a function of sulfhydryl residues reacted with DTNB. The experimental conditions were the same as described for Figure 1. For details regarding the determination of sulfhydryl residues reacted with DTNB, see section on Materials and Methods. The course of activity loss was followed by withdrawing aliquots of the reaction mixture at different time intervals and assaying them in the standard system as described under Materials and Methods.

Importance of these two residues in the function of this enzyme was also verified by an experiment in which the enzyme was inactivated by titration with stoichiometric amounts of DTNB. Though some inactivation occurred even at very small ratios of DTNB to enzyme (<1), most of the inactivation resulted only on the addition of the second mole of DTNB. This showed that inactivation is primarily the result of reaction of about 1 mol of DTNB with a mol of enzyme.

It was also observed that addition of each mole of DTNB to the enzyme resulted in the release of two TNB anions. This showed that during the inactivation process two sulfhydryl residues were simultaneously modified via the formation of an inter- or intramolecular disulfide bond (Glazer, 1970). This result is thus compatible with that described in Figure 3 to the extent that two sulfhydryl residues get modified during the inactivation process.

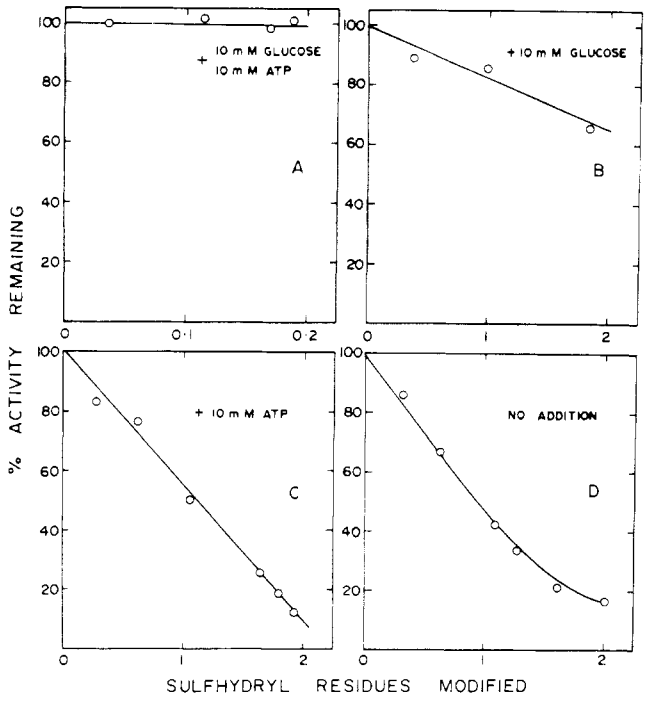

FIGURE 4: Sulfhydryl residues modified and activity lost on addition of 1 mol of DTNB to a mol of disulfide containing hexokinase derivative in presence and absence of substrates. Concentration of hexokinase was 3.8 μM in 50 mM Tris-chloride buffer (pH 8.0) containing 0.5 mM EDTA. Loss of activity and sulfhydryl modification were monitored as already described. Times of reaction are as follows: (A and B) 2 hr; (C and D) 45 min.

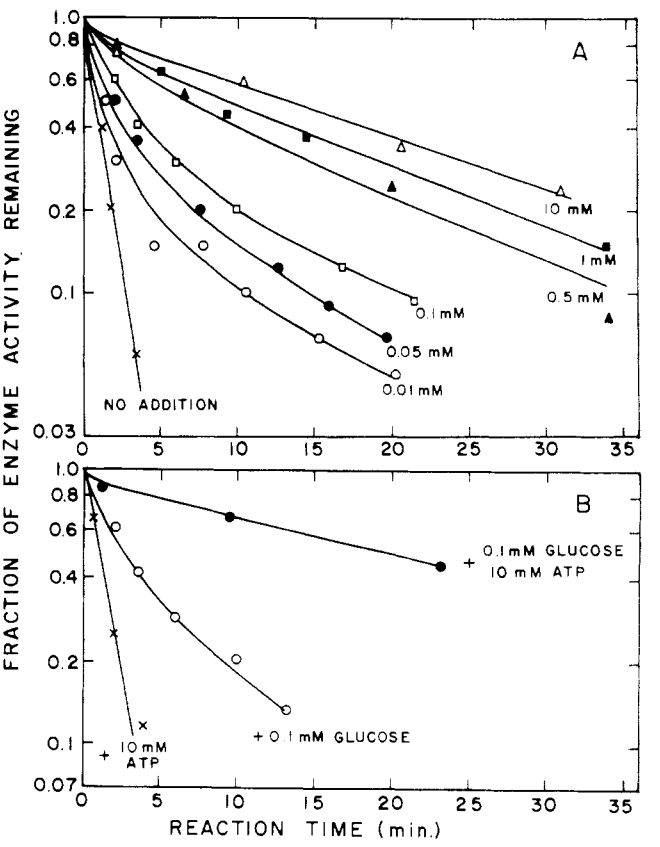

FIGURE 5: Inactivation of hexokinase by DTNB in presence of substrates. 2.8 μM hexokinase in 50 mM Tris-chloride buffer (pH 8.0) containing 0.5 mM EDTA was inactivated with 0.5 mM DTNB in presence of substrates as indicated. (A) Inactivation of hexokinase in presence of varying concentrations of glucose as shown on the curves. (B) Inactivation of hexokinase in presence of 0.1 mM glucose and/or 10 mM ATP. Additions are shown on the curves.

A clear demonstration that the inactivation of the enzyme required the modification of only two sulfhydryl residues presumably at the glucose binding site of the enzyme was provided by the following experiment.

Hexokinase dialyzed in Tris-EDTA buffer was treated with 1 mol of DTNB per mol of enzyme in presence of saturating concentrations (10 mM) of glucose and ATP. The reaction was carried out in presence of substrates so as to prevent a fraction of DTNB from attacking the thiols at the substrate binding sites. Practically no fall in activity was noted but two TNB anions were released indicating the formation of a disulfide bond in the enzyme. The two sulfhydryls participating in this disulfide bond were presumably the fast reacting class A residues not involved in enzyme activity (Table I; Figure 3). The presence of the disulfide bond in this fully active enzyme derivative was confirmed by determining the free sulfhydryl content of this derivative before and after treatment with mercaptoethanol.

For the purpose of the experiment, the hexokinase derivative containing the disulfide bond was then exhaustively dialyzed to remove glucose, ATP, and the TNB anions. The dialyzed enzyme had about 80% of the activity of the original material. The dialyzed preparation was divided into four portions; 10 mM glucose and 10 mM ATP were added to the first portion, 10 mM glucose to the second, 10 mM ATP to the third, and to the last nothing was added. All of these portions were next treated with 1 mol of DTNB per mol of enzyme and the loss in enzyme activity was correlated with the reaction of sulfhydryl residues as monitored by the release of the TNB anions. The reaction was carried out for about 2 hr or till about two TNB anions were released whichever was earlier. The results are shown in Figure 4A–D. In presence of glucose plus ATP there is no loss in enzyme activity and negligible reaction with sulfhydryl residues even over a 2-hr period. (Figure 4A). In presence of glucose alone, about 35% activity is lost and about two sulfhydryl residues get modified in 2 hr (Figure 4B). On the other hand, in the presence of ATP alone or in the absence of any ligand, nearly all the enzyme activity is lost rather rapidly in 45 min and a very satisfactory correlation can be established between complete inactivation and reaction of two sulfhydryl residues to form a disulfide bond (Figure 4C and D).

Some of the other conclusions that are consistent with the data are as follows. Since no inactivation occurs in presence of glucose plus ATP, it is possible that DTNB inactivates the enzyme by reacting at both or at either of the substrate binding sites. Since in the presence of ATP alone or in the absence of any ligand almost total inactivation occurs and about two sulfhydryl residues get modified to form a disulfide bond, one may argue with some reason that these residues are at the glucose binding site of the enzyme (also see text relating to Table I). However, these results can also be satisfactorily explained by assuming that a conformational change occurs in the enzyme on interaction with glucose for which some evidence already exists (Table I).

The slow inactivation that occurs in presence of glucose is completely prevented when ATP is present in addition to glucose. This is an intriguing result because ATP by itself does not afford any protection. Also when ATP is present in addition to glucose, not only is there no loss in activity but also negligible reaction of DTNB with sulfhydryl residues of the enzyme (Figure 4A). Since two sulfhydryl residues

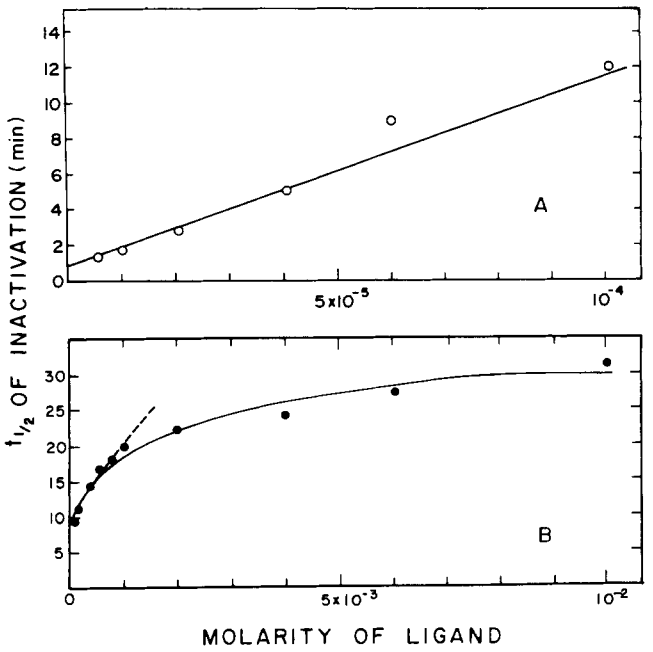

FIGURE 6: Dissociation constants of glucose and ADP with respect to the enzyme. Dissociation constants were calculated on the basis of eq 3. (A) Dissociation constant of glucose. For this experiment 0.36 m*M* DTNB was used to inactivate 2.8 μM hexokinase. (B) Dissociation constant of ADP. For this experiment 0.1 m*M* DTNB was used to inactivate 2.8 μM hexokinase.

do react in presence of glucose alone or ATP alone (Figures 4B and C), their failure to react with DTNB in simultaneous presence of these ligands points to a possible synergism in the interaction of these ligands with the enzyme. This cooperative interaction between these substrates perhaps gives rise to a hexokinase molecule whose sulfhydryl residues do not react significantly with DTNB. Needless to say, this hypothesis does not require the presence of sulfhydryl residues at the substrate binding sites, although it does not rule out this possibility.

Experiments that follow appear to support this idea of synergism and interdependence in the binding of substrates to brain hexokinase.

Protection by Ligands against Inactivation of the Enzyme by DTNB and Determination of Enzyme/Ligand Dissociation Constants. Figure 5A illustrates the protective effect of glucose against inactivation of the enzyme by excess DTNB followed kinetically. The figure shows that the protection by glucose increases with its concentration till saturation is reached at about 1 m*M* concentration. It must be noted that though in the complete absence of glucose, the enzyme is inactivated by DTNB in a monophasic fashion, at nonsaturating concentrations of glucose (<0.1 m*M*), the inactivation process is clearly biphasic. This indicates that glucose binding causes a slow conformational change in the enzyme which is irreversible during the time course of the experiments. This would result in a partitioning of the enzyme into two independent species, one native and the other modified, which are inactivated at different rates by DTNB.

Figure 5B shows that though a fully saturating concentration of 10 m*M* ATP provides no protection to the enzyme against inactivation by DTNB (also see Table I and Figure 4C), the biphasic character of inactivation kinetics in presence of 0.1 m*M* glucose is completely abolished when 10 m*M* ATP is present in addition to glucose. The enzyme too is protected much more strongly against inactivation by the reagent in the simultaneous presence of these two ligands. This suggests that in presence of 10 m*M* ATP, glucose enforces a kind of conformation on the enzyme which is different from that present in the absence of ATP. The reactivity of the sulfhydryl residues of the enzyme toward DTNB is also drastically curtailed when these ligands are present together at a fully saturating concentration of 10 m*M* (Table I). Since this reduction in reactivity cannot be easily explained by the additive effects of the two ligands, a synergism in their interaction with hexokinase is strongly indicated.

This conclusion was further tested by saturating one of the substrate sites and analyzing the protective effect of the other substrate against the inactivating action of the reagent. The enzyme was saturated with 10 m*M* ATP, and kinetics of its inactivation by DTNB was followed at various levels of glucose. Using excess DTNB, the decay in enzyme activity was found to be monophasic. The protective effect of glucose against the inactivation of the enzyme-ATP complex by DTNB could be analyzed by treating glucose as a competitive inhibitor of the inactivation reaction. We have already shown in a previous section that inactivation of the enzyme by DTNB resembles enzyme-substrate interaction (eq 1). Following Dixon and Webb (1964), and assuming that the site to which glucose is bound in the enzyme-ATP complex cannot react with DTNB

$$k_{obsd} = \frac{k_2}{1 + \frac{(K_{(DTNB,ATP)}}{[DTNB])}\left(1 + \frac{[glucose]}{K_{glucose}}\right)} \quad (2)$$

Here k_{obsd} is the observed pseudo-first-order rate constant of the inactivation reaction in presence of glucose, k_2 is as defined in eq 1, $K_{glucose}$ is the dissociation constant of glucose with respect to the enzyme-ATP complex, and $K_{(DTNB,ATP)}$ is the dissociation constant of DTNB with respect to enzyme saturated with ATP.

The reciprocal form of the above equation is

$$\frac{1}{k_{obsd}} = \frac{1}{k_2}\frac{K_{(DTNB,ATP)}}{[DTNB]} + \frac{1}{k_2} + \frac{1}{k_2}\frac{K_{(DTNB,ATP)}}{[DTNB]}\frac{[glucose]}{K_{glucose}} \quad (3)$$

Thus on plotting $1/k_{obsd}$ (or $t_{1/2}$ of inactivation) for inactivation of the enzyme by DTNB against concentration of glucose, a straight line is obtained as shown in Figure 6A. From the intercept, $1/k_2(1 + K_{(DTNB,ATP)}/[DTNB])$, it is easy to calculate $1/k_2$ since $K_{(DTNB,ATP)}$ and [DTNB] are known. It follows from eq 3 that when $1/k_{obsd} = 1/k_2$, [glucose] = $-K_{glucose}$. From Figure 6A, $K_{glucose} = 0.11 \times 10^{-5}$ *M* which is very much lower than the kinetically determined K_m of glucose with respect to the enzyme (Table II). The reason for this discrepancy is not clear.

The reverse experiment in which the enzyme is saturated with glucose (10 m*M*) and its inactivation by DTNB followed at various levels of ATP also gave monophasic kinetics. Using the value of 10^{-4} *M* as the dissociation constant of DTNB with respect to the enzyme saturated with glucose $K_{(DTNB,glucose)}$, the dissociation constant of ATP with respect to enzyme-glucose complex, K_{ATP}, is calculated to be 1.2×10^{-4} *M* (plot not shown).

The protective effect of Glc-6-P against inactivation of the enzyme by DTNB is complete at 10 m*M* concentration of the ligand (Table I). At lower concentrations of this li-

Table II. Michaelis, Inhibitor, and Dissociation Constants for Interaction between Type I Hexokinase and Various Ligands.

Ligand	K_m (M)	K_i (M)	K_d by the Method Described here (M)	K_d by Other Methods (M)
Glucose	3.7×10^{-5} [a]		0.11×10^{-5}	
	4.5×10^{-5} [b]			3.0×10^{-5} [e]
ATP	5.0×10^{-4} [a]		1.2×10^{-4}	
	4.0×10^{-4} [b]			2.2×10^{-4} [e]
Glc-6-P		2.6×10^{-5} [b]	1.1×10^{-5}	
		1.2×10^{-5} [c]		0.7×10^{-5} [d]
ADP		0.6×10^{-3} [b]	0.5×10^{-3}	
P_i			0.8×10^{-3}	0.25×10^{-3} [d]

[a] Data of Joshi and Jagannathan (1968). [b] Data of Grossbard and Schimke (1966). [c] Data of Ning et al. (1969). [d] Data of Wilson (1973). [e] Data of Ellison et al. (1974).

gand the kinetics of this inactivation is biphasic. As in the case of glucose (Figure 5), this biphasic kinetics may be due to a slow conformational change induced in the enzyme by Glc-6-P which is relatively irreversible during the time course of the experiment. When glucose at 10 mM concentration is added to this system, the biphasic kinetics disappears and the enzyme is inactivated in a monophasic fashion indicating some effect of glucose on the interaction of Glc-6-P with the enzyme. The data also showed that whereas 0.1 mM Glc-6-P protects very little and 10 mM glucose protects moderately, both together protect almost completely. Using the same method as already illustrated in the case of ATP, the dissociation constant of Glc-6-P with respect to enzyme–glucose complex is found to be 1.1×10^{-5} M.

The results presented above had shown that in presence of 10 mM glucose, both ATP and Glc-6-P behaved in a parallel fashion in protecting the enzyme and in abolishing biphasic inactivation kinetics. This was indicative of a common site on the enzyme for the binding of Glc-6-P and ATP. Fromm and Zewe (1962), on the basis of their kinetic experiments, had suggested that Glc-6-P is anchored to the enzyme at the γ-phosphate site of ATP. This idea receives strong support from an experiment in which the enzyme was partially protected against inactivation by DTNB with 0.8 mM Glc-6-P. When 10 mM ATP was added to this system, the protective effect of Glc-6-P almost completely vanished indicating that Glc-6-P was displaced by ATP. A similar effect was also seen with P_i but not with ADP, indicating that P_i and Glc-6-P occupy the same site on the enzyme as the γ phosphate of ATP.

The data on the reactivity of sulfhydryl residues in presence of ADP, ATP, or P_i had indicated that all these three ligands bind to overlapping sites on the enzyme (Table I). It was thus considered possible that ADP and P_i would also provide strong protection to the enzyme in presence of glucose. This was shown by saturating the enzyme with 10 mM glucose and following the kinetics of its inactivation with DTNB at varying levels of P_i or ADP. It was found that both P_i and ADP responded in the same way as ATP or Glc-6-P in presence of fully saturating levels of glucose indicating that glucose in some way modifies the binding sites of P_i and ADP to the enzyme. The protective effect of P_i or ADP was much less than that with ATP or Glc-6-P. Using the same procedure as employed in case of ATP, the dissociation constant of P_i with respect to glucose–enzyme complex was found to be 0.8×10^{-3} M.

An anomalous situation was observed with ADP. Though the protective effect of ADP increased with rising concentration of ADP up to a concentration of 1 mM, any further increase caused no corresponding increase in the protective effect (Figure 6B). In fact at 50 mM concentration of ADP, a reversal of the protective effect could be seen (experiment not shown). The plot in Figure 6B clearly shows a deviation from linearity at higher concentrations of ADP. Extrapolating from the linear section of this plot representing lower concentrations of the ligand, the dissociation constant for ADP with respect to the enzyme–glucose complex was found to be 0.5×10^{-3} M. The aberrant behavior of ADP as shown by the nonlinearity of the plot in Figure 6B suggests a two-site binding of ADP to the enzyme as proposed by Ning et al. (1969).

If our suggestion that ATP, ADP, and P_i occupy a common site on the enzyme is right, then ATP should be displaced by ADP or P_i. Since as pointed out above, the protective effect of ADP and P_i in presence of glucose is less than that of ATP, one should be able to see less protection when ADP or P_i displaces ATP. As expected, it was found that the protective effect of ATP in presence of glucose is diminished when ADP or P_i are further added to the system (data not shown).

The dissociation constants for the various ligands obtained by the method described here are compared in Table II with their kinetic and dissociation constants obtained by other methods.

Discussion

The results presented in this paper provide strong evidence that two fast reacting sulfhydryl residues of the enzyme (class A) are not required for enzyme activity but that two residues of class B reactivity are important for enzyme function and are probably located at the glucose binding site. Essential sulfhydryl residues with similar reactivity have also been identified by Chou and Wilson (1974) in rat brain hexokinase.

Our data leave open the question whether the inactivation of the enzyme is primarily the result of a disulfide bridge introduced in the enzyme by DTNB or the mixed disulfide intermediate between the enzyme and DTNB formed prior to it (Glazer, 1970). Also the formation of a disulfide bond was detected by using limiting amounts of DTNB, a condition which actually contributes to its formation. At relatively high concentrations of DTNB employed in most of the experiments reported here, it is quite possible that mixed disulfides rather than disulfide bridges are formed. Alternatively it is possible that the mixed disulfide of the enzyme with the TNB moiety may still be an active species as observed by Flashner et al. (1972) in the case of muscle pyruvate kinase.

The disulfide bridges formed in the enzyme on addition of stoichiometric amounts of DTNB appeared to be mostly intramolecular on the basis of the sedimentation behavior of the enzyme so treated. However, the formation of a few intermolecular disulfide bridges during the process of inactivation cannot be ruled out as about 30% of this enzyme sedimented as an aggregate (patterns not shown).

It is also interesting to note that addition of up to 4 mol of DTNB per mol of enzyme in the absence of substrates resulted in the formation of about four disulfide bridges as shown by the release of eight TNB anions (results not shown). It was not possible to follow the stoichiometry of TNB release beyond this stage as the rate of reaction became very slow. However, the formation of these four disul-

fide bridges leads us to believe that most of the sulfhydryl residues of the enzyme are clustered fairly close together.

Inactivation of hexokinase by DTNB was found to be pseudo-first-order with respect to enzyme sites even at only tenfold excess of the reagent over the enzyme. Considering the fact that the enzyme has about 12 sulfhydryl residues (Redkar and Kenkare, 1972), it is clear that the DTNB shows remarkable selectivity in attacking residues crucial for enzyme activity. This phenomenon is explained by our observation that DTNB binds to the sites containing the sulfhydryls prior to the modification reaction. Our data showing competition between DTNB and the substrates to bind to the same site on the enzyme resemble the results obtained by Kamikashi et al. (1974) with *p*-chloromercuribenzoate.

The slow conformational changes that we invoke in brain hexokinase in presence of glucose and Glc-6-P have been well documented both in yeast (Shill and Neet, 1971) and in mammalian hexokinases (Kosow et al., 1973). The interdependence of glucose and ATP binding sites that we postulate in brain hexokinase has its parallel in yeast hexokinase where Colowick and coworkers have shown the requirement of glucose for ATP binding and have also suggested the possibility of ATP promoting glucose binding (Colowick, 1973). More recently Fletterick et al. (1975) have reported that whereas glucose can bind only one subunit of the yeast hexokinase dimer, ATP requires glucose for binding and then promotes binding of glucose to the second subunit. Also our observations indicate that brain hexokinase–glucose–ATP ternary complex has an entirely different type of conformation compared to the binary enzyme–substrate complexes. A similar situation has been shown to exist with regards to complexes yeast hexokinase forms with glucose and Mg-ATP (Shill et al., 1974).

The problem of the order of addition of substrates to the enzyme presents an enigma. Though our results strongly suggest that binding sites for glucose and ATP are interdependent, there are also sufficient data to indicate that glucose and ATP bind to brain hexokinase independently of each other as suggested by Ning et al. (1969) and Bachelard et al. (1971). Thus both our results (Table I; Redkar and Kenkare 1972) as well as those of Chou and Wilson (1974) on rat brain hexokinase show changes in sulfhydryl reactivity when glucose or ATP is separately added to the enzyme system. We do not know how to reconcile these two sets of data but it would be useful to consider the results obtained by Fletterick et al. (1975) on yeast hexokinase. These authors found that there are two sets of sites for the binding of nucleotides to yeast hexokinase, one dependent on glucose binding and the other independent of it. It is tempting to speculate that in brain hexokinase too, there are two types of ATP binding sites, one glucose dependent and the other glucose independent.

It is also interesting to note that all the effects of nucleotides ATP and ADP could be obtained in the absence of the magnesium ion indicating that the metal is not required for binding of nucleotides to hexokinase. Similar results have been reported with rat brain hexokinase (Chou and Wilson, 1974). Brain hexokinase may thus be similar to creatine kinase (Cohn, 1963) in the way the nucleotides and the metal react with the enzyme.

Our inference that ATP and ADP as well as ATP and Glc-6-P have overlapping sites on the enzyme surface is easily explained on the principle of compulsory overlap of substrates and products at a catalytic center. However, our conclusion regarding the identity of the binding site for P_i, Glc-6-P, and γ phosphate of ATP would need some comment. Thus our suggestion that P_i and γ phosphate of ATP bind at a common site is in conflict with reports from other laboratories (Purich et al., 1973; Kosow et al., 1973) that P_i had no effect on the K_m of ATP for the enzyme. We have no satisfactory explanation for this discrepancy unless the presence of Mg^{2+} in kinetic experiments makes the difference. Regarding our contention that P_i and Glc-6-P share the same site on the enzyme, it may be pointed out that Kosow et al. (1973) have shown that P_i acts competitively with respect to Glc-6-P in human erythrocyte hexokinase type I and Wilson (1973) has indicated that both P_i and Glc-6-P compete for a single regulatory site on rat brain hexokinase. On the other hand, Ellison et al. (1974) have proposed a common site for ATP and Glc-6-P but another site for P_i on the bovine brain enzyme. As already mentioned above, we interpret our results as indicating a common site for P_i, Glc-6-P, and ATP.

Acknowledgments

We thank the reviewers of this paper and Professor S. P. Colowick for their constructive criticism regarding interpretation of part of the data presented here. Thanks are also due to Mr. B. Subbarao for stimulating discussions.

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Kinetics of Sulfate Transport by *Penicillium notatum.* Interactions of Sulfate, Protons, and Calcium†

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ABSTRACT: The active transport of inorganic sulfate by an ATP sulfurylase-negative strain of *Penicillium notatum* is promoted by H^+ ions and metal ions (divalent metal ions being more effective than monovalent metal ions). Initial velocity studies suggest that H^+ and SO_4^{2-} add to the carrier in an ordered sequence (H^+ before SO_4^{2-}), with H^+ at equilibrium with free carrier and carrier-H^+ complex. The linear reciprocal plots and replots suggest a 1:1 stoichiometry between H^+ and SO_4^{2-}. Ca^{2+} and other divalent metal ions stimulate sulfate transport markedly in buffered suspensions of low ionic strength. The kinetics of the Ca^{2+}/SO_4^{2-} interaction suggest that Ca^{2+} (like H^+) adds to the carrier before SO_4^{2-} and is at equilibrium with free carrier and carrier-Ca^{2+} complex. The linear reciprocal plots and replots indicate a 1:1 stoichiometry between Ca^{2+} and SO_4^{2-}. Thus the fully loaded carrier-SO_4^{2-}-Ca^{2+}-H^+ complex has a net positive charge relative to that of the free carrier, a fact consistent with the chemiosmotic hypothesis of membrane transport. The kinetics of the H^+/Ca^{2+} interaction point to a random A-B (rapid equilibrium), ordered C sequence with A = H^+, B = Ca^{2+}, and C = SO_4^{2-}. Selenate (an alternate substrate competitive with sulfate) is an uncompetitive inhibitor with respect to Ca^{2+}, in agreement with the suggested mechanism. Internal charge balance is not accomplished by a stoichiometric coaccumulation of Ca^{2+} and SO_4^{2-}. Sulfate transport does, however, promote $^{45}Ca^{2+}$ uptake. A significant fraction of the added Ca^{2+} is bound by the mycelial surface. Binding is extremely rapid, but reversible.

Penicillium notatum and related species possess a number of distinct, well-characterized membrane transport systems (Benko et al., 1967, 1969; Bellenger et al., 1968; Tweedie and Segel, 1970; Skye and Segel, 1970; Hackette et al., 1970; Bradfield et al., 1970; Hunter and Segel, 1971; Goldsmith et al., 1973). All of these systems display an obvious pH optimum. In some systems (e.g., amino acid transport), the effect of pH can be related in part to the concentration of the proper ionic form of the substrate (Hunter and Segel, 1971). In other systems (e.g., inorganic sulfate, nitrate, and choline-*O*-sulfate transport), the substrate exists in the same ionic form throughout the pH range tested. In these cases, H^+ must be affecting some component of the transport system. The simplest explanation is that the carrier must exist in the proper ionic form in order to bind and/or translocate the substrate. Thus, a study of the effect of pH on the kinetics of substrate transport may shed some light on the nature of the chemical group(s) at the carrier active site.

The effect of pH on transport rates can be interpreted in another way: there is a good deal of evidence that active membrane transport in microorganisms is energized by a proton and/or charge gradient across the cell membrane (interior higher pH and/or negative) (Harold, 1972; Hunter and Segel, 1973; Seastron et al., 1973; Slayman and Slayman, 1974). According to this view, a membrane carrier is, in fact, a proton carrier that possesses an additional binding site for a particular solute. H^+ moves inward in response to the membrane potential and the solute is carried along against a concentration gradient. Whether we accept this chemiosmotic explanation for active transport or not, it is possible to apply the principles of enzyme kinetics and consider H^+ and the specific solute as cosubstrates of a bireactant system. We can then ask two questions. What is the order of addition of H^+ and the solute to the carrier? What is the stoichiometry between H^+ and the solute (i.e., how many molecules of each combine with the carrier)? The answers to the above questions were the major objectives of this study.

The sulfate transport system of *Penicillium* and *Aspergillus* species differs from others that we have studied in that transport requires a relatively high ionic strength of the incubation medium (Bradfield et al., 1970). In contrast, the fungi will transport neutral and positively charged substrates (e.g., amino acids, choline-*O*-sulfate, and methylammonium) from deionized water (Hackette et al., 1970). The high ionic strength might simply be required to induce the proper conformational state of the sulfate carrier. If this is true, any combination of nontoxic ions should promote

† From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616. *Received May 20, 1975.* This work was supported by Grant BMS74-13675 from the National Science Foundation.