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Na^+,K^+-ATPase: Ligand-Induced Conformational Transitions and Alterations in Subunit Interactions Evidenced by Cross-Linking Studies†

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ABSTRACT: The membrane-bound Na^+,K^+-ATPase is an oligomer containing several α subunits (catalytic subunits) and several β subunits of unknown function. To obtain information on subunit interactions, we studied the effects of the enzyme's physiological ligands on the cross-linking of the α subunits in the presence of *o*-phenanthroline and Cu^{2+}. Two distinct cross-linking patterns were observed depending on the concentrations of the cross-linking reagents. (1) In the presence of 0.25 mM Cu^{2+} and 1.25 mM *o*-phenanthroline, cross-linking did not occur unless ATP was added. This ATP-induced formation of the cross-linked α,α dimer was stimulated by Na^+ and inhibited by K^+. The half-maximal effect was obtained at 2–5 μM ATP. Na^+ + ATP dependent dimer formation was accompanied by Na^+-dependent phosphorylation of the enzyme. The steady-state level of phosphoenzyme was attained within 10 s, while the level of the α,α dimer continued to rise up to 5 min. The formation of phosphorylated dimer and the conversion of this to unphosphorylated dimer by K^+ were demonstrated. ADP and the β,γ-methylene analogue of ATP did not induce dimer formation. The exposure of a set of sulfhydryl groups upon phosphorylation of α subunit and subsequent dimerization are suggested by the data. (2) In the presence of 0.25 mM Cu^{2+} and 0.5 mM *o*-phenanthroline, a cross-linked α,α dimer was obtained in the absence of Na^+, K^+, and ATP and under all ligand conditions except when K^+ + ATP was added. The half-maximal inhibitory effect of ATP on dimer formation in the presence of K^+ was obtained at ~0.2 mM ATP. In the presence of K^+, ADP and the β,γ-methylene analogue of ATP also inhibited dimer formation. These data suggest the existence of a conformational state of the α subunit with bound K^+ and ATP (at a low-affinity site) and with a set of occluded sulfhydryl groups. When either ligand is removed, the groups are exposed and dimerization occurs. Because the conformational transitions detected by the cross-linking studies are known to be closely related to Na^+ and K^+ translocation steps, the findings suggest that alterations in subunit interactions at a domain of two α subunits regulate the transport function of the enzyme.

The active transports of Na^+ and K^+ across the plasma membranes of most eucaryotic cells are carried out by Na^+,K^+-ATPase.[1] The early studies on the complex kinetics of the reactions catalyzed by the enzyme (Robinson, 1967) and on the different reactive states of the phosphorylated enzyme (Albers, 1967; Post et al., 1969) suggested the occurrence of a variety of ligand-induced conformational transitions of the enzyme, and this has been supported by a multitude of subsequent studies along similar lines (Glynn & Karlish, 1975; Albers, 1976). More direct evidence for such transitions has been obtained with the aid of several conformational probes (Hart & Titus, 1973; Jorgensen, 1975; Karlish et al., 1978; Karlish & Yates, 1978). Examination of this literature indicates, as expected, that the conformational states revealed by one probe may not be distinguished by another. It is clear, therefore, that if the correlation of the various conformational transitions with the transport function of the enzyme is to be attempted, different probes must be used.

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[1] Abbreviations used: Na^+,K^+-ATPase, sodium- plus potassium-dependent adenosinetriphosphatase (EC 3.6.1.3); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate.

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It has been established that the highly purified Na^+,K^+-ATPase contains two polypeptides in association with the lipids of the membrane (Glynn & Karlish, 1975; Albers, 1976). One is the catalytic subunit (α subunit) with a molecular weight of ~100 000, and the other (β subunit) is a glycoprotein of uncertain role with a molecular weight of ~40 000. A proteolipid with a molecular weight of ~12 000, and with unknown function, may also be a part of the enzyme (Forbush & Hoffman, 1978; Rogers & Lazdunski, 1979). Studies on the molecular weight of the detergent-solubilized enzyme (Hastings & Reynolds, 1979; Esmann et al., 1979) suggest that the functional unit of the enzyme contains several α subunits and β subunits. The subunit stoichiometry is not known. The exploration of the quaternary structure of the enzyme has also been attempted through cross-linking studies (Kyte, 1975; Giotta, 1976; Liang & Winter, 1977; Sweadner, 1977; Huang & Askari, 1978, 1979a). The results of these studies are also consistent with the existence of two or more of each subunit in the native state. Because the oligomeric nature of Na^+,K^+-ATPase seems to have been established, general considerations pertaining to conformational aspects of enzyme regulation (Koshland, 1970) suggest that at least some of the ligand-induced conformational transitions within a subunit may be reflected in alterations in subunit interactions.

In a recent report (Huang & Askari, 1979b) we presented data indicating that the chemical cross-linking of the α subunits of the enzyme in the presence of Cu^{2+} and *o*-phenanthroline is influenced by Na^+, K^+, and ATP and we suggested that such cross-linking experiments may provide a novel approach to the study of the ligand-induced conformational transitions of the enzyme. The continuation of this work is described here. The data suggest that with the proper choice of ligands and cross-linking conditions, three different conformational states of the enzyme may be distinguished: a phosphorylated form of the enzyme, the dephosphoenzyme with K^+ and ATP bound to it, and the dephosphoenzyme in any form but that containing bound K^+ and ATP. If the reasonable assumption is made that the chemical cross-linking of the subunits is an indication of the noncovalent association of the subunits in the native state, the findings may also be interpreted in the context of ligand-induced alterations in subunit interactions and the relation of such interactions to the transport function of the enzyme.

Experimental Procedures

Na^+,K^+-ATPase was obtained from dog kidney outer medulla by the "rapid" version of the purification of Jorgensen (1974). It should be noted that this involves the selective removal of unwanted membrane components by $NaDodSO_4$ and results in a highly purified but still membrane-bound enzyme. The specific activities, as defined and measured by Jorgensen (1974), of the various preparations used were in the range of 18–26 units/mg. The enzyme was stored in 0.25 M sucrose, 30 mM histidine, and 1 mM Tris–EDTA (pH 6.8). Immediately prior to use it was sedimented by centrifugation at 100 000*g* for 1 h and washed twice with 50 mM Tris-HCl (pH 7.4) to remove histidine and EDTA. For all cross-linking and phosphorylation studies the enzyme at a final concentration of 0.5 mg/mL was suspended in a solution containing 10 mM Tris-HCl (pH 7.4) and the indicated concentrations of NaCl, KCl, $MgCl_2$, nucleotides, and the cross-linking reagents. All experiments were done at 24 °C.

When the experiment was done for the purpose of detecting the formation of cross-linked products, the reaction was terminated by the addition of EDTA to a final concentration of 30 mM, followed by $NaDodSO_4$ to obtain a final detergent concentration of 5% (Huang & Askari, 1979b). Aliquots were then subjected to $NaDodSO_4$–polyacryamide gel electrophoresis (Huang & Askari, 1978). The stained gels were photographed or scanned photometrically. For quantitative determination of products, peak heights in photometric scans were compared. In selected experiments the results obtained in this way were compared with those obtained by the measurement of area under each curve. No significant differences were noted. All such cross-linking experiments were done for a fixed period of 10 min, except the experiments of Figure 6 which were done for the periods indicated in the legend to the figure.

The phosphorylation experiments with γ-labeled [^{32}P]ATP were carried out for the indicated periods and were terminated by the addition of an equal volume of cold 8% $HClO_4$. After centrifugation, the pellets were washed twice with 0.1% trichloroacetic acid containing 10 mM inorganic phosphate and 1 mM unlabeled ATP. The pellets were either counted by conventional procedures or dissolved in $NaDodSO_4$ and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis, at pH 2.4, by the procedure of Avruch & Fairbanks (1972). For measurement of the ATPase activity, released inorganic phosphate from γ-labeled [^{32}P]ATP was converted to phosphomolybdate, extracted into 2-methylpropanol, and counted (Henderson & Askari, 1977).

Results

Cross-Linked Products. We have demonstrated before (Huang & Askari, 1978) that when the enzyme is reacted either with Cu^{2+} or with *o*-phenanthroline and Cu^{2+} a variety of cross-linked products of the two subunits of the enzyme may be obtained and that the relative amounts of these products are governed by the duration of the experiment and by the concentrations of the enzyme and the cross-linking reagent. Concerning the experiments to be described here, it is important to note the following. (1) Conditions have been standardized (constant enzyme concentration and constant and short reaction time) such that the formation of products with molecular weights higher than those of dimers of the subunits are minimized. (2) In all experiments the major cross-linked products are an α,α dimer and an α,β dimer. These products are identified on the basis of their mobilities and staining characteristics (Liang & Winter, 1977; Huang & Askari, 1978). (3) The cross-linked α,β dimer is not a covalently linked product and is easily dissociated in the presence of EDTA (Huang & Askari, 1978). Because we have not detected any ligand effects on the formation of the α,β dimer (Huang & Askari, 1979b), in all experiments to be described here this product has been eliminated from the gels by the addition of EDTA to the reaction mixture after the completion of the cross-linking reaction and prior to the electrophoresis of the sample (see Experimental Procedures). (4) The present experiments deal primarily with the formation of cross-linked α,α dimer. It will be noted below that this dimer is formed under several different cross-linking conditions: with Cu^{2+} alone; with Cu^{2+} and *o*-phenanthroline; with Cu^{2+}, *o*-phenanthroline, and various combinations of the enzyme's physiological ligands. In experiments similar to those indicated before (Huang & Askari, 1978), we have found that regardless of the cross-linking condition the resulting α,α dimer is resistant to EDTA and dissociable in mercaptoethanol and that the formation of the α,α dimer under all conditions is prevented if the enzyme is pretreated with *N*-ethylmaleimide. These findings suggest that the dimer is the result of the covalent cross-linking of two α subunits through a disulfide bond. We have also mixed the α,α dimer bands obtained under the

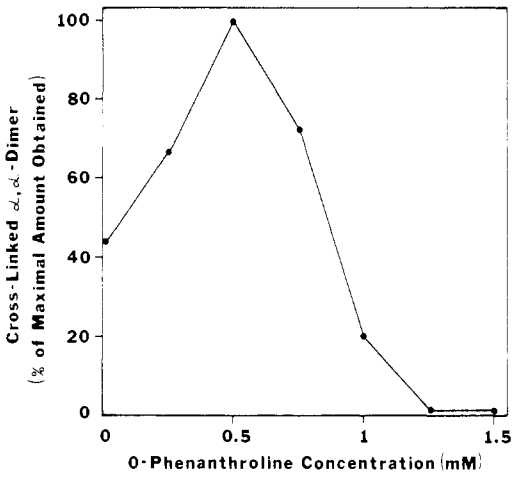

FIGURE 1: Effects of varying concentrations of *o*-phenanthroline on the formation of cross-linked α,α dimer in the presence of 0.25 mM $CuSO_4$. The cross-linking reaction, subsequent gel electrophoresis, and measurement of the extent of dimer formation were performed as described in the text.

different cross-linking conditions, subjected the mixtures to electrophoresis on gels containing different acrylamide concentrations, and have obtained single bands in all cases. In spite of these superficial similarities, there is no compelling reason to believe that the same sulfhydryl groups are involved in the cross-linking of the α subunits under the various cross-linking conditions. In fact, the experiments presented below suggest that some of the α,α dimers obtained under different conditions may not be identical products.

Cross-Linking in the Presence of Cu^{2+} and o-Phenanthroline. The experiments of Figure 1 show the effects of varying concentrations of *o*-phenanthroline and a fixed concentration of Cu^{2+} (0.25 mM) on the formation of the cross-linked α,α dimer. We should note that in the absence of *o*-phenanthroline when Cu^{2+} concentrations lower than 0.25 mM were used, the amounts of the dimer were lower than that obtained with 0.25 mM Cu^{2+}. Thus, the stimulatory effect of *o*-phenanthroline on cross-linking noted in Figure 1 cannot be explained by the lowering of the free Cu^{2+} concentration that results upon addition of *o*-phenanthroline. The data indicate that cross-linking is being induced by a complex of *o*-phenanthroline and Cu^{2+}. *o*-Phenanthroline is known to form 1:1, 2:1, and 3:1 complexes with Cu^{2+} (James & Williams, 1961). The complex responsible for cross-linking cannot be identified precisely from the data. Because increasing concentrations of *o*-phenanthroline eventually lead to complete inhibition of cross-linking (Figure 1), it is reasonable to assume that cross-linking must be induced by either the 1:1 complex, or the 2:1 complex, or both. It is of interest to note that the oxidation of a simple sulfhydryl compound, 5,5′-dithiobis(2-nitrobenzoic acid), by *o*-phenanthroline and Cu^{2+} seems to be catalyzed by the 2:1 complex (Kobashi, 1968).

Lack of Ligand Effects on Cross-Linking in the Presence of Cu^{2+}. We have already mentioned (Huang & Askari, 1979b) that the formation of cross-linked α,α dimer in the presence of Cu^{2+}, without *o*-phenanthroline, is not affected by the enzyme's physiological ligands. For the interpretation of the experiments on ligand effects in the presence of Cu^{2+} and *o*-phenanthroline, it is necessary to describe the experiments with Cu^{2+} alone in more detail. Under the standard cross-linking conditions used here, the amount of α,α dimer formed in the presence of 0.25 mM Cu^{2+} (Figure 1) is the maximal amount that can be obtained in the absence of *o*-phenanthroline. With 0.05 mM Cu^{2+} the amount of dimer formed is less than 20% of that obtained with 0.25 mM Cu^{2+}. Utilizing these two concentrations of Cu^{2+}, one optimal and one suboptimal, we have examined the effects of varying concentrations of Na^+ (1–100 mM), K^+ (0.1–25 mM), and ATP (0.1–1 mM) on the formation of the α,α dimer. We have found that the ligands, used individually and in various combinations, have no effects on cross-linking under these conditions.

Ligand Effects on Cross-Linking in the Presence of Cu^{2+} and o-Phenanthroline. In our previous report (Huang & Askari, 1979b) we showed that when *o*-phenanthroline and Cu^{2+} are used in a molar ratio of 2:1, a condition under which the maximal amount of α,α dimer is formed (Figure 1), the following effects of Na^+, K^+, and ATP are obtained. When both K^+ and ATP are present in the reaction mixture, the α,α dimer is not obtained. Dimer formation is not affected, however, when any of the ligands is added alone or when the ligands are added in any combination other than K^+ + ATP. Subsequent experiments revealed significant changes in the above ligand effects when the relative concentrations of Cu^{2+} and *o*-phenanthroline were altered. Eventually, it was determined that two distinct patterns of ligand effects on cross-linking are obtained: one as described above and the other when the ratio of *o*-phenanthroline to Cu^{2+} is so high that no cross-linking is obtained in the absence of the enzyme's physiological ligands (Figure 1). Under the latter conditions the following effects of ligands are observed. (1) The formation of the α,α dimer is induced upon addition of ATP. (2) Na^+ stimulates the ATP-dependent dimer formation. (3) K^+ inhibits the ATP-dependent dimer formation. For comparison of the above two patterns of ligand effects, at high and low *o*-phenanthroline concentrations, on cross-linking, see Figure 2 of this paper and Figure 1 of Huang & Askari (1979b). The remainder of the experiments of this paper deals with the more detailed analysis of these cross-linking reactions and with the relationship between the cross-linking patterns and the various ligand–enzyme interactions.

Reactions Occurring in the Presence of 0.25 mM Cu^{2+} and 1.25 mM o-Phenanthroline. As evident from Figure 2 when *o*-phenanthroline and Cu^{2+} are used in the molar ratio of 5:1, the presence of ATP, either with or without Na^+, is necessary for the induction of cross-linking. We already know that the presence of *o*-phenanthroline is also mandatory. As indicated above, when Cu^{2+} without *o*-phenanthroline is used at concentrations that are suboptimal for cross-linking, the addition of ATP is without any effect. Another trivial possibility, namely, that the combination of ATP and *o*-phenanthroline may be sufficient for cross-linking, was ruled out by doing experiments with wide ranges of ATP and *o*-phenanthroline concentrations in the absence of Cu^{2+}. Clearly, the minimum requirement for cross-linking under the indicated conditions is the simultaneous presence of ATP, Cu^{2+}, and *o*-phenanthroline.

Figure 3 shows the effects of varying concentrations of ATP, ADP, CTP, ITP, and the β,γ-methylene analogue of ATP on cross-linking. Figures 4 and 5 show the stimulatory effects of varying concentrations of Na^+ and the inhibitory effects of varying concentrations of K^+ on ATP-induced cross-linking. From the data of Figures 3–5 the following points are evident.

(1) The apparent K_m of ATP for the induction of cross-linking is 2–5 μM (Figure 3). This is approximately the same as the apparent K_m values of ATP for Na^+-dependent phosphorylation of the enzyme in the presence of Mg^{2+} (Kanazawa et al., 1970; Post et al., 1965) and for Na^+-dependent ATPase activity (Kanazawa et al., 1967; Neufeld & Levy, 1969). It

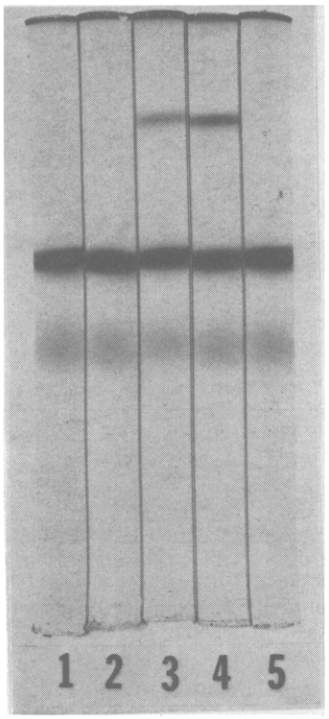

FIGURE 2: Effects of ATP, Na^+, and K^+ on the formation of cross-linked α,α dimer in the presence of 0.25 mM Cu^{2+} and 1.25 mM *o*-phenanthroline. Enzyme samples were incubated with the cross-linking reagent and the indicated ligands as described in the text and then subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. The three bands from top to bottom are α,α dimer, α monomer, and β monomer. Gel 1, control enzyme without cross-linking; gel 2, incubated with the cross-linking reagent in the absence of ligands; gel 3, 1 mM ATP; gel 4, 1 mM ATP + 100 mM Na^+; gel 5, 1 mM ATP + 25 mM K^+. Gels from samples incubated with Na^+ or K^+ in the absence of ATP are not shown. They were the same as gels 1, 2, and 5.

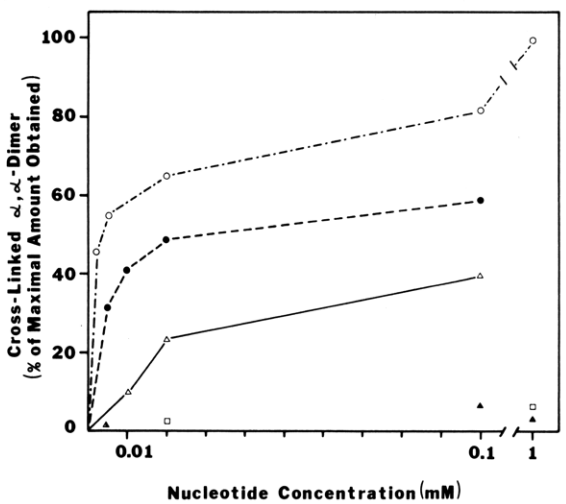

FIGURE 3: Effects of varying concentrations of ATP (○), CTP (●), ITP (△), ADP (▲), and the β,γ-methylene analogue of ATP (□) on the formation of cross-linked α,α dimer in the presence of 0.25 mM Cu^{2+}, 1.25 mM *o*-phenanthroline, and 100 mM Na^+. The results are expressed as the percentage of the amount of dimer obtained with 1 mM ATP.

is slightly higher than the dissociation constant of the enzyme's high-affinity ATP binding site (0.1–0.3 μM) as determined by equilibrium binding studies (Hegyvary & Post, 1971; Norby & Jensen, 1971). However, the dissociation constants determined by binding studies were obtained in the absence of divalent cations and there is evidence to indicate that Mg^{2+} reduces the apparent affinity of the nucleotides for this site

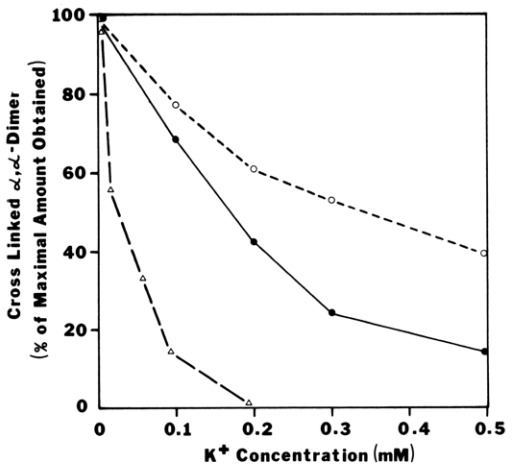

FIGURE 4: Inhibitory effects of varying concentrations of K^+ on the formation of cross-linked α,α dimer in the presence of 0.25 mM Cu^{2+} and 1.5 mM *o*-phenanthroline. (○) 100 mM Na^+ + 1 mM ATP; (●) 100 mM Na^+ + 0.01 mM ATP; (△) 1 mM Na^+ + 1 mM ATP. For each set the results are expressed as the percentage of the amount of dimer obtained in the absence of K^+.

(Kaniike et al., 1973; Mårdh & Post, 1977). If we assume that Cu^{2+} or *o*-phenanthroline and Cu^{2+} may have an effect similar to that of Mg^{2+} (an assumption that will be supported by experiments presented below), it is reasonable to conclude that the effect of ATP in cross-linking experiments is due to its binding to the same high-affinity site that is detected in binding studies and in studies on Na^+-dependent phosphorylation and Na^+-dependent ATPase activity.

(2) The order of effectiveness of the nucleotides (ATP > CTP > ITP) in cross-linking experiments (Figure 3) is the same as that observed for the binding of nucleotides to the high-affinity site (Hegyvary & Post, 1971; Jensen & Norby, 1971). However, because ADP and the β,γ-methylene analogue of ATP do not induce cross-linking (Figure 3) but both are known to bind to the high-affinity nucleotide site (Jensen & Norby, 1971), we may suspect that it is not the binding of the nucleotide but rather the subsequent phosphorylation of the enzyme that induces cross-linking.

(3) The effects of Na^+ and K^+ and the kinetics of these effects on ATP-induced cross-linking are consistent with the above conclusion. The antagonistic effects of K^+ and ATP (Figure 4) are similar to those observed in studies on the binding of ATP to the enzyme (Hegyvary & Post, 1971; Norby & Jensen, 1971). The apparent competitive effects of K^+ and and Na^+ (Figure 4) are similar to those observed in studies on ATP binding (Hegyvary & Post, 1971; Norby & Jensen, 1971), on Na^+-dependent phosphorylation of the enzyme (Mårdh, 1975), and on Na^+-dependent ATPase activity at low ATP concentrations (Neufeld & Levy, 1969; Henderson & Askari, 1977). And the synergistic effects of Na^+ and ATP (Figure 5) are consistent with a similar phenomenon observed in phosphorylation studies (Mårdh & Post, 1977). Also, considering the crudeness of the measurements on cross-linking experiments in comparison with those of ligand binding and enzyme kinetic studies, there is gratifying agreement between the apparent K_m values of Na^+ and K^+ in the cross-linking experiments (Figures 4 and 5) and the similar values observed in the above-cited studies.

On the basis of the above considerations, the possibility of a critical role of the phosphoenzyme in ATP-induced cross-linking was tested through the following experiments.

The experiments of Table I were done to compare the normal pattern of the phosphorylation of the enzyme by ATP in the presence of Mg^{2+} with the results obtained when Mg^{2+}

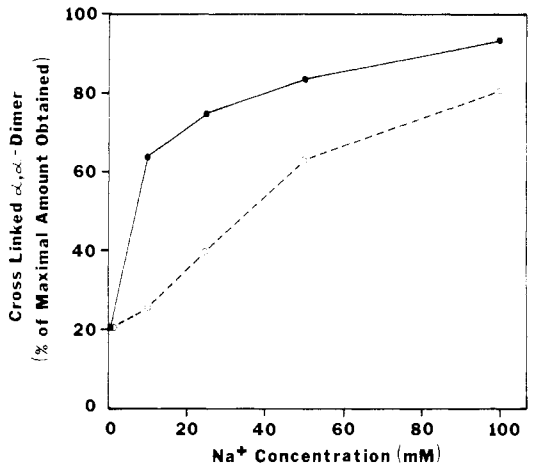

FIGURE 5: Stimulatory effects of varying concentrations of Na^+ on the formation of cross-linked α,α dimer in the presence of 0.25 mM Cu^{2+}, 1.5 mM *o*-phenanthroline, 0.1 mM K^+, and ATP (O, 0.01 mM ATP; ●, 1 mM ATP). The results are expressed as the percentage of the amount of dimer that was obtained in the presence of 1 mM ATP and 100 mM Na^+ and no K^+.

Table I: ATPase Activity and Phosphorylation by 0.3 mM [^{32}P]ATP of the Enzyme in the Presence of 2 mM Mg^{2+} and in the Presence of 1.25 mM *o*-Phenanthroline and 0.25 mM Cu^{2+}

reaction conditions[a]	^{32}P incorpn (nmol/mg) 10 s	30 s	ATPase act. (nmol of P_i per mg per min)[b]
Mg^{2+}	1.40	1.38	35
Mg^{2+} + 100 mM Na^+	6.50	6.30	178
Mg^{2+} + 10 mM K^+	1.21	1.21	24
o-phenanthroline-Cu^{2+}	4.70	4.55	37
o-phenanthroline-Cu^{2+} + 100 mM Na^+	6.63	6.70	42
o-phenanthroline-Cu^{2+} + 10 mM K^+	1.20	1.30	24
Cu^{2+} + 100 mM Na^+	1.45	1.30	25
Cu^{2+} + 10 mM K^+	1.25	1.20	31

[a] Other reaction conditions are described in the text. [b] This value is calculated on the basis of P_i released at 10 s.

is replaced with Cu^{2+} and *o*-phenanthroline. Release of inorganic phosphate in the course of the experiment was also measured. It should be noted that these experiments were done with the same concentrations of enzyme, Cu^{2+}, and *o*-phenanthroline and at the same temperature as the cross-linking experiments of Figures 3–5. The data indicate that (a) the maximal steady-state level of labeling of the enzyme is obtained either with Mg^{2+} + Na^+ or with Cu^{2+} + *o*-phenanthroline + Na^+, (b) in the absence of monovalent cations the extent of labeling in the presence of Cu^{2+} + *o*-phenanthroline is greater than that in the presence of Mg^{2+} but less than the maximal level that is obtained in the presence of Na^+, (c) the increment of labeling obtained in the presence of Cu^{2+} + *o*-phenanthroline is inhibited by K^+, and (d) when Mg^{2+} is replaced with Cu^{2+} + *o*-phenanthroline, little or no Na^+-dependent ATPase activity occurs.

A portion of the experiments of Table I involving *o*-phenanthroline and Cu^{2+} was repeated with the use of [^{14}C]ATP instead of [^{32}P]ATP. The results ruled out the possibility that the data of Table I may be due to the tight binding of intact ATP to the enzyme. Bound ATP, if any, was less than 10% of the maximal ^{32}P incorporation and was not influenced by Na^+ and K^+.

Comparison of the data of Table I with those of Figure 2 suggests that when ATP-dependent cross-linking occurs, the

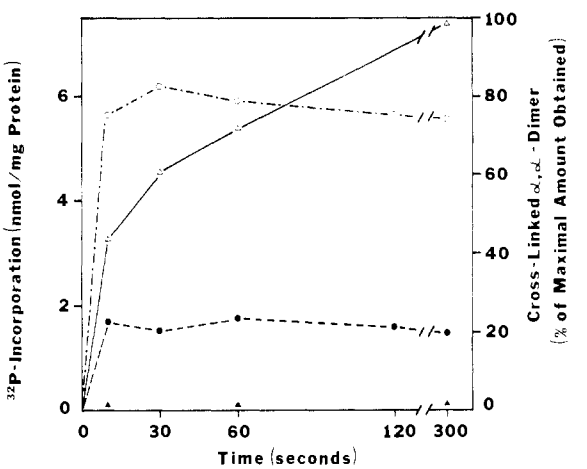

FIGURE 6: Comparison of the time course of the formation of phosphoenzyme with that of the formation of α,α dimer. Enzyme samples were incubated in the presence of 0.25 mM Cu^{2+}, 1.25 mM *o*-phenanthroline, 0.3 mM ATP, and either 100 mM Na^+ (O and △) or 10 mM K^+ (● and ▲). In one set γ-labeled [^{32}P]ATP was used, and the total level of ^{32}P incorporation (O and ●) was measured as described in the text. In the other set unlabeled ATP was used, and the level of α,α dimer formed (△ and ▲) was measured as described in the text.

phosphoenzyme is also formed. It is not certain whether cross-linking and phosphorylation obtained in the absence of Na^+ are because of the presence of low levels of bound Na^+ in the enzyme preparation, due to the contamination of the cross-linking reagents with Na^+, or due to the Na^+-like effect of Cu^{2+} and *o*-phenanthroline in the process of formation of the phosphoenzyme.

The experiments of Figure 6 were done to compare the time course of the formation of the α,α dimer in the presence of Na^+ + ATP + Cu^{2+} + *o*-phenanthroline with the time course of the labeling of the enzyme under the same conditions. It is evident that while the maximal level of the phosphoenzyme is obtained within 10 s and maintained thereafter, the level of the cross-linked dimer is continuously rising during the experiment. These data show that phosphoenzyme formation and cross-linking do not occur simultaneously and suggest that cross-linking is a slower process that follows the phosphorylation of the enzyme.

A logical question arising from the above experiments is whether or not the cross-linked α,α dimer is phosphorylated. The experiments of Figure 7 show that it is. Here the enzyme was phosphorylated and cross-linked in the presence of Na^+, ATP, *o*-phenanthroline, and Cu^{2+} and was then subjected to electrophoresis at pH 2.4. It is evident that ^{32}P incorporation into both the α-monomer peak and the α,α-dimer peak has occurred. The data also show the expected effect of the replacement of Na^+ with K^+.

The data of Figure 8 provide limited information on the relative stabilities and K^+ sensitivities of the phosphorylated cross-linked α,α dimer and the phosphorylated enzyme that is not cross-linked. The former seems to be more stable than the latter when phosphorylation and cross-linking are terminated by the addition of EDTA. The discharge of both phosphorylated species is, however, stimulated upon addition of K^+. Incidentally, comparison of the data of Figure 8 with those of Figure 6 shows that the effect of K^+ on cross-linking is because of prevention of cross-linking rather than due to stimulation of the breakdown of the α,α dimer.

Reactions Occurring in the Presence of 0.25 mM Cu^{2+} and 0.5 mM o-Phenanthroline. When *o*-phenanthroline and Cu^{2+} are used in a molar ratio of 2:1, cross-linking occurs under all

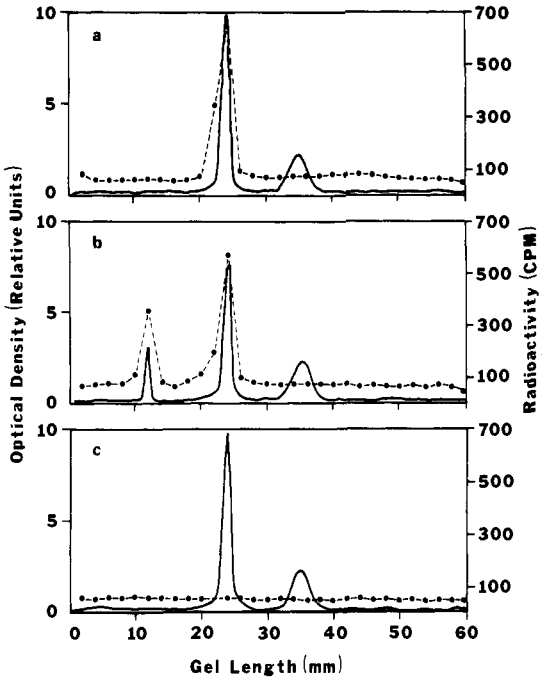

FIGURE 7: ^{32}P incorporation into cross-linked α,α dimer. Enzyme samples were phosphorylated with 0.3 mM γ-labeled [^{32}P]ATP for 60 s in the presence of (a) 2 mM Mg^{2+} and 100 mM Na^+, (b) 0.25 mM Cu^{2+}, 1.25 mM *o*-phenanthroline, and 100 mM Na^+, and (c) 0.25 mM Cu^{2+}, 1.25 mM *o*-phenanthroline, and 10 mM K^+. Reactions were terminated by the addition of $HClO_4$, and samples were subjected to gel electrophoresis at pH 2.4 as described in the text. The three peaks, from left to right, are α,α dimer, α monomer, and β monomer. Optical density is indicated by the solid line and radioactivity by the dashed line.

Table II: Effects of 50 and 300 μM ATP on Enzyme Phosphorylation and the Formation of Cross-Linked α,α Dimer in the Presence of 0.25 mM Cu^{2+} and 0.5 mM *o*-Phenanthroline[a]

	^{32}P incorpn (nmol/mg)		α,α dimer (% of maximal amount obtained)		
reaction conditions	50 μM ATP	300 μM ATP	no ATP	50 μM ATP	300 μM ATP
o-phenanthroline-Cu^{2+} + 100 mM Na^+	5.55	6.50	100	100	100
o-phenanthroline-Cu^{2+} + 10 mM K^+	0.85	1.90	100	100	22
2 mM Mg^{2+} + 100 mM Na^+	5.64	6.65			
2 mM Mg^{2+} + 10 mM K^+	1.01	2.05			

[a] Control phosphorylation reactions in the presence of Mg^{2+} were done for 10 s. Reactions with *o*-phenanthroline and Cu^{2+} were carried out for 2 min. A portion of each sample was used for the measurement of ^{32}P incorporation, and another portion was used for gel electrophoresis as described in the text.

ligand conditions except when both K^+ and ATP are present. In our previous report (Huang & Askari, 1979b) we indicated that prevention of cross-linking under these conditions requires relatively high concentrations of ATP and that the apparent K_m of ATP for this effect is about the same as the apparent K_m of ATP for the low-affinity nucleotide site of the K^+ form of the enzyme (Post et al., 1972; Jorgensen, 1975). The following experiments strengthen the assumption that it is the binding of ATP to this low-affinity site rather than its interaction with the high-affinity site involved in enzyme phosphorylation that is responsible for the prevention of cross-linking under these conditions.

The experiments of Figure 9 were done to see if changes in the concentrations of the cross-linking reagents affect the

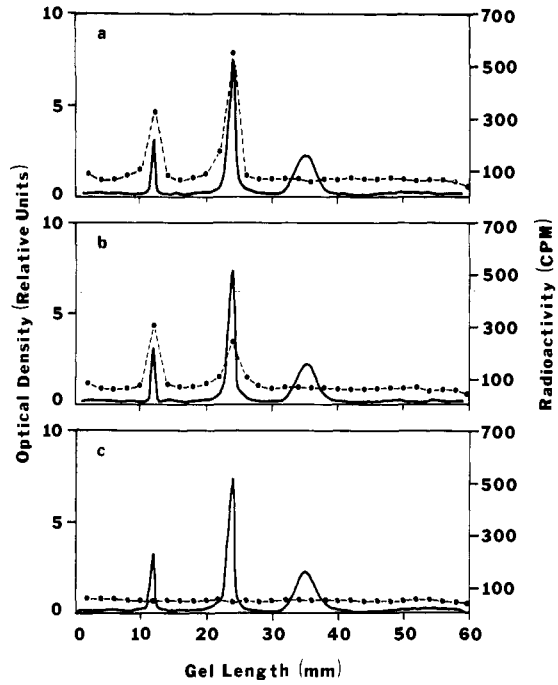

FIGURE 8: Effect of K^+ on ^{32}P-labeled α,α dimer. Three enzyme samples were phosphorylated and cross-linked as described in the legend to Figure 7 in the presence of 0.25 mM Cu^{2+}, 1.25 mM *o*-phenanthroline, and 100 mM Na^+. After 60 s, (a) the reaction was terminated by the addition of $HClO_4$, (b) EDTA was added to a final concentration of 10 mM, incubation was continued for an additional 10 s, and $HClO_4$ was then added, and (c) 10 mM EDTA and 10 mM K^+ were added simultaneously, incubation was continued for 10 s, and $HClO_4$ was added.

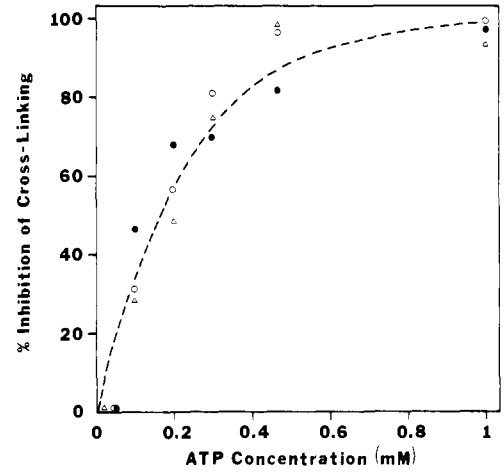

FIGURE 9: Inhibitory effects of varying concentrations of ATP and 10 mM K^+ on the formation of cross-linked α,α dimer in the presence of (○) 0.25 mM Cu^{2+} and 0.5 mM *o*-phenanthroline, (●) 0.5 mM Cu^{2+} and 1 mM *o*-phenanthroline, and (△) 0.1 mM Cu^{2+} and 0.2 mM *o*-phenanthroline.

apparent K_m of ATP. In three sets of experiments different concentrations of reagents were used, but in all cases the molar ratio of *o*-phenanthroline to Cu^{2+} was 2:1. It is evident that the concentration of ATP for the half-maximal effect is about the same (0.2 mM) in all three experiments and that in no case is cross-linking prevented at ATP concentrations of 50 μM or below.

In the experiments of Table II cross-linking and enzyme phosphorylation in the presence of 0.25 mM Cu^{2+} and 0.5 mM *o*-phenanthroline were compared at two different ATP concentrations. In the presence of 50 μM ATP and K^+ cross-linking is not prevented, whereas with 300 μM ATP and K^+

considerable inhibition of cross-linking is obtained. At both ATP concentrations, however, Na^+-stimulated and K^+-inhibited phosphorylation are observed. Obviously, there are no correlations between the occurrence and prevention of phosphorylation on the one hand and the occurrence and prevention of cross-linking on the other.

In our previous report (Huang & Askari, 1979b) we stated that ADP + K^+ prevents cross-linking that is induced by 0.25 mM Cu^{2+} and 0.5 mM *o*-phenanthroline. Additional experiments showed that the 1 mM β,γ-methylene analogue of ATP also prevents cross-linking under these conditions. It is clear that here the nucleotide specificity is different from that observed in the experiments of Figure 3.

Effects of Mg^{2+}. In experiments similar to those of Figure 2 it was found that the addition of 2 mM Mg^{2+} has little or no influence on the cross-linking patterns obtained either in the presence of 0.25 mM Cu^{2+} and 0.5 mM *o*-phenanthroline or in the presence of 0.25 mM Cu^{2+} and 1.25 mM *o*-phenanthroline. Whether or not Mg^{2+} has subtle effects on the kinetics of various ligand effects on cross-linking has not been determined.

Enzyme Activity after Cross-Linking. When the enzyme was incubated under the various cross-linking conditions indicated above, washed to remove excess reagents, and then assayed, partial inhibition of the $Na^+ + K^+$ dependent ATPase activity was observed. In most experiments, the extent of inhibition was greater than that which may be accounted for by the extent of formation of inactive cross-linked products. This confirms the findings of others (Giotta, 1976; Liang & Winter, 1977) and suggests the occurrence of intramolecular cross-linking of sulfhydryl groups which cannot be detected by the methods used. Studies on the characteristics of the residual activity are in progress.

Discussion

The major constraints under which the interpretation of the present findings must be attempted are the general shortcomings of cross-linking studies as structural probes of membrane proteins (Peters & Richards, 1977). Briefly, these are (a) the uncertainties concerning the nature of the reactions, the identities of the products, and the significance of the yields of the various products, (b) the relative crudeness of the existing analytical methods for both qualitative and quantitative determinations of the products, and (c) the inability to distinguish with certainty between collision and stable complexes. Hence, some of the discussions that follow are speculative and the conclusions that are reached must be considered to be tentative.

For the moment, let us set aside the matter of the enzyme's quaternary structure. The data presented here show that a reaction or a series of reactions of the enzyme with Cu^{2+} and *o*-phenanthroline leading to the apparent oxidation and dimerization of the α subunit is influenced by Na^+, K^+, and ATP. It is rather clear-cut that the selective effects of Na^+ and K^+ on cross-linking patterns must be due to cation interactions with the enzyme. This is indicated by the general similarities between the kinetics of Na^+ and K^+ effects on cross-linking and the kinetics of cation interactions with the enzyme studied through a variety of other approaches (Glynn & Karlish, 1975; Albers, 1976). The roles of Cu^{2+}, *o*-phenanthroline, and ATP in the cross-linking reactions, however, are more difficult to analyze.

In the absence of the enzyme's physiological ligands, maximal cross-linking occurs when the molar ratio of *o*-phenanthroline to Cu^{2+} is 2:1 and cross-linking is prevented when this ratio is increased to 5:1. The most reasonable explanation for this phenomenon is that excess *o*-phenanthroline causes the disappearance of that species of *o*-phenanthroline–Cu^{2+} complex (most likely the 2:1 complex) which catalyzes the oxidation of a set of the enzyme's sulfhydryl groups. Under the latter conditions (excess *o*-phenanthroline), the addition of 2–5 μM ATP is sufficient to induce the half-maximal level of cross-linking. Binary complexes of Cu^{2+} and ATP and ternary complexes of Cu^{2+}, ATP, and *o*-phenanthroline have been characterized (Mitchell & Sigel, 1978). The ATP-induced cross-linking cannot be due to an ATP–Cu^{2+} complex because we know that the presence of *o*-phenanthroline is necessary. The concentration of added ATP, compared to those of Cu^{2+} and *o*-phenanthroline, is so small that is not likely that ATP is exerting its effect by creating significant changes in the relative concentrations of the various complexes of Cu^{2+} and *o*-phenanthroline. A likely explanation is that the ATP-induced cross-linking is due to a ternary complex of ATP, Cu^{2+}, and *o*-phenanthroline, though the independent interactions of the three ligands or their binary complexes with the enzyme cannot be ruled out. In this regard, we should note that even for the normal reactions of the enzyme in which Mg^{2+} and ATP are involved, it is difficult to say whether free Mg^{2+} and free ATP or MgATP, or the combination of these interacts with the enzyme (Skou, 1974; Robinson, 1974). In any case, phosphorylation studies in the presence of ATP, 0.25 mM Cu^{2+}, and 1.25 mM *o*-phenanthroline suggest that ATP, in some form, interacts with the high-affinity active site of the enzyme and that this leads to an Na^+-stimulated phosphorylation of the α subunit that precedes the formation of the cross-linked α,α dimer. The fact that the dimer, which continues to accumulate after the maximal level of phosphorylation has been attained, is also phosphorylated indicates that the phosphorylated α subunit participates in the cross-linking reaction. Previous studies on the interaction of *N*-ethylmaleimide with the enzyme (Hart & Titus, 1973) have already indicated that the phosphorylation of the enzyme in the presence of Mg^{2+}, ATP, and Na^+ exposes a set of sulfhydryl groups to *N*-ethylmaleimide. Thus, it is reasonable to propose that (a) a similar conformational transition occurs after phosphorylation in the presence of ATP, Cu^{2+}, and *o*-phenanthroline and (b) the oxidation of the exposed sulfhydryl groups is catalyzed by Cu^{2+} already bound in the vicinity, resulting in the formation of intermolecular disulfide bonds among two α subunits. The opposing effects of Na^+ and K^+ on ATP-induced cross-linking and on phosphorylation are consistent with this proposal.

When *o*-phenanthroline and Cu^{2+} are used in a molar ratio of 2:1, the cross-linking that is presumably catalyzed by an *o*-phenanthroline–Cu^{2+} complex occurs regardless of whether the enzyme is phosphorylated or not. The simplest way of explaining these observations is that the sulfhydryl groups oxidized under these conditions are different from those that are exposed under phosphorylation. The formation of the cross-linked α,α dimer under these conditions is prevented when both K^+ and high concentrations of ATP are present. This suggests the existence of a stable conformational state of the enzyme with bound K^+ and ATP (at a low affinity site) containing a set of occluded sulfhydryl groups that becomes exposed upon the removal of either K^+ or ATP. The occurrence of an enzyme species with bound K^+ and ATP at a low-affinity site has been suggested by several past studies [e.g., Hegyvary & Post (1971) and Skou (1979)]. The previously used conformational probes (Jorgensen, 1975; Karlish et al., 1978; Karlish & Yates, 1978) have indicated that the binding of ATP to the low-affinity site accelerates the conversion of

the K^+ form of the enzyme to the Na^+ form. The present findings provide the additional information that this conversion proceeds through a conformational state with bound K^+ and ATP that is distinct from both the K^+ form and the Na^+ form. It is evident from the above discussions that in spite of the complexities of the cross-linking reactions under study, such experiments may be utilized as probes of conformational transitions of the enzyme.

The above conclusions were reached without the consideration of the status of the quaternary structure of the enzyme. As we have discussed elsewhere (Huang & Askari, 1979a), in spite of the shortcomings of cross-linking experiments as probes of quaternary structures of membrane proteins (Peters & Richards, 1977), the cumulative evidence from a variety of studies makes it reasonable to assume that the formation of the cross-linked α,α dimer is indeed indicative of the existence of a stable noncovalent α oligomer in the native enzyme. Now let us examine the additional information that is gained if the interpretation of the data is attempted in the context of this assumption.

(1) As indicated already, it is apparent that under certain cross-linking conditions it is the phosphorylated α subunit, with its newly exposed sulfhydryl groups, that is involved in cross-linking. There are two possible explanations for this. One is that a phosphorylated α subunit is being cross-linked to an unphosphorylated α subunit. This may be rationalized by assuming that the unmasking of the relevant sulfhydryl groups occurs not only in the phosphorylated subunit but also in the adjacent unphosphorylated subunit because of the changes in subunit interactions that occur upon phosphorylation. The more intriguing possibility is that two phosphorylated subunits are being cross-linked. If this is so, since most available evidence indicates that under optimal conditions no more than half of the α subunits may be phosphorylated (Glynn & Karlish, 1975), we must conclude that the native enzyme contains at least a tetramer of the α subunit. The possibility of the existence of more than two α subunits in the native membrane-bound enzyme has been raised (Skou & Esmann, 1979). It seems, therefore, that the clarification of whether two phosphorylated subunits are being cross-linked or one phosphorylated subunit is being cross-linked to an unphosphorylated one may be of help in the determination of the number of α subunits in the native state. In principle, it should be possible to distinguish between the above alternatives through the measurement of the extent of ^{32}P incorporation into α,α dimer and α monomer in experiments such as those of Figure 7. However, for such calculations more extensive information on the stabilities of the two phosphorylated forms and on the relative yields of the products in the course of experiments is needed. It is hoped that the issue will be resolved by the experiments that are in progress.

(2) The conformational states that are detectable by cross-linking experiments are associated with the formation of the phosphoenzyme and the formation of a form of enzyme containing bound K^+ and ATP. As discussed by Karlish et al. (1978), several studies on Na^+–Na^+ exchange, uncoupled flux of Na^+, and K^+–K^+ exchange catalyzed by Na^+,K^+-ATPase in intact cells suggest that the outward movement of Na^+ is associated with the conformational transitions of the phosphorylated enzyme and that the enzyme with bound K^+ and ATP is connected with the release of K^+ at the inner surface of the membrane. Thus, it seems that the conformational states detected by cross-linking are related to the two steps within the complex reaction sequence of the enzyme that are most intimately related to the translocations of Na^+ and K^+ across the membrane. If the existence of an α oligomer is prerequisite for the formation of the cross-linked α,α dimer, we may conclude that the conformational transitions most closely associated with ion translocations must be accompanied by significant alterations in subunit interactions at the domain of two α monomers. The attractiveness of this seemingly benign conclusion is that it fits rather well with those models of the pump in which ion translocations across the membrane are envisioned to occur through a channel between two α subunits that spans the membrane (Singer, 1974; Kyte, 1975). The possibility arises, therefore, that cross-linking experiments may be a way of looking at alterations in the geometry of the transport channel during a cycle of the Na^+,K^+ pump.

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Proton Magnetic Resonance Study of Crambin, a Hyperstable Hydrophobic Protein, at 250 and 600 MHz†

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ABSTRACT: Crambin is a 44 amino acid, molecular weight 5000, water-insoluble protein of such crystalline thermal ordering that it yields a high-resolution X-ray diffraction pattern to the interplanar spacing of 0.88 Å [Teeter, M. M., & Hendrickson, W. A. (1979) *J. Mol. Biol. 127*, 219–223]. The protein represents a unique model system to investigate the physical properties of hydrophobic polypeptides, ubiquitous in biological membranes. Crambin has been studied by using 250- and 600-MHz ^{1}H NMR spectroscopy. Organic solvents of intermediate polarity had to be used to dissolve the protein in a homogeneous phase. We find that both acetic acid, a weak Brønsted proton donor, and dimethylformamide, a weak Lewis nucleophile, perform satisfactorily in preserving essentially identical globular structures. The whole aliphatic spectrum exhibits a high degree of nondegeneracy, indicating a rather rigid structure. Most of the 24 methyl peaks can be accounted for as they yield relatively sharp signals within an ~1.4-ppm range, well dispersed at a magnetic field of 14.09 T. The phenylalanyl and the two tyrosyl side chains are well resolved and show line widths and resonance frequencies indicative of immobilization. In acetic-d_3 acid-*d*, crambin exhibits such a remarkable low rate of ^{1}H–^{2}H exchange that, at room temperature, deuteration is still incomplete after 20 days in solution. By this criterion, a minimum of 28 to 29 peptidyl amides are shown to be solvent protected, which reflects a large extent of intramolecular hydrogen bonding and sheltered locations. A few stable amide signals appear at field positions higher than the aromatic resonances, suggesting that a number of NH groups are occluded in a hydrophobic matrix. All of these features indicate that crambin is a protein of unprecedented structural stability.

Although high-resolution nuclear magnetic resonance (NMR)[1] spectroscopy is the method of choice for the study of conformational and dynamic features of proteins in solution, its application to such problems is often limited by the size of the molecule or its state of aggregation. Even with the present day availability of high-field spectrometers, the technique is of limited usefulness beyond M_r ~10–15 K as the spectra become extremely complex in terms of the number of mostly overlapping signals and much of the fine structure information is lost due to dipolar line broadening. This poses a serious problem in the study of hydrophobic proteins which are normally water insoluble and tend to aggregate when dissolved in less polar solvent systems.

Because of their fundamental role as components of biological membranes, hydrophobic polypeptides are increasingly becoming the focus of attention when attempting to understand basic cellular processes. However, due to the water insolubility that these proteins exhibit, structural studies have thus far been mostly limited to diffraction techniques of various sorts, centered on crystalline or highly ordered lamellar systems. It is clear that the wealth of information provided by such approaches would be well supplemented if dynamic features, relevant to their physiological roles, could also be investigated by, e.g., NMR spectroscopy. Although this appears to be a reasonable goal, the first requirement to be met is the need to solubilize the protein in a suitable, nondenaturing solvent that preserves the protein as a monomeric unit.

Crambin is an M_r 5000 hydrophobic protein found in the seed of the plant *Crambe abyssinica* (Van Etten et al., 1965). It has the peculiarity of readily crystallizing out of aqueous ethanol solutions in space group $P2_1$ needles that diffract X-rays strongly to the interplanar spacing limit of 0.88 Å; the resolution thus afforded is characteristic of crystals from small organic molecules (Van Etten et al., 1965; Teeter & Hen-

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[1] Abbreviations used: DMF, dimethylformamide-d_7; Me_2SO, dimethyl-d_6 sulfoxide; M_r, molecular weight; NMR, nuclear magnetic resonance; ppm, parts per million; Me_4Si, tetramethylsilane; F_3AcOD, trifluoroacetic acid-d_1.

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