Cooperative Effects of Substrates and Substrate Analogs on the Conformation of Creatine Phosphokinase*

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ABSTRACT: The rate of inhibition of creatine phosphokinase by iodoacetamide or *p*-nitrophenylacetate is reduced in the presence of an equilibrium mixture of the substrates of the enzyme: $MgATP^{2-}$, creatine, $MgADP^{-}$, and phosphocreatine. It has now been shown that the addition of the nonfunctional substrate pair, $MgADP^{-}$ and creatine, is necessary and sufficient for this protection. This observation has allowed a more direct evaluation of previously reported substrate-dependent conformational changes in this enzyme. Studies of sedimentation constant, reduced viscosity, deuterium exchange, and trypsin susceptibility indicate only small structural alternations in the protein can be related to the binding of the combined substrates.

Samuels *et al.* (1961) have reported that creatine phosphokinase (CPK[1]) undergoes a substrate-dependent change in optical rotation which was interpreted as reflecting a conformational change in the protien. This change was reported to be dependent upon the simultaneous presence of both substrates, and it was suggested that it might play an important role in the mechanism of enzyme action. This hypothesis of a special configuration for "working enzyme" has been employed by Watts and Rabin (1962) to explain the protection against carboxymethylation with iodoacetamide of the two reactive sulfhydryl groups of the enzyme which is afforded by the substrate mixture. Little protection was obtained if either $MgATP^{2-}$ or creatine alone was present. Similarly, Watts (1963) has reported that *p*-nitrophenylacetate inhibits CPK and that the substrate mixture gives strong protection against this inhibition. Since we have recently shown (Clark *et al.*, 1964) that *p*-nitrophenylacetate inhibition results from acetylation of the enzyme at a site different from the reactive cysteine residues, it would appear that the effects of substrate binding are felt over an appreciable area of the enzyme protein structure.

In order to define better the limits of substrate protection, as well as to assess the magnitude of the postulated conformational change, we have undertaken a comparison of the effects of several substrates and substrate analogs, in various combinations, upon (a) the inhibition of CPK by iodoacetamide and by *p*-nitrophenylacetate, (b) the susceptibility of CPK toward proteolytic degradation by trypsin, and (c) the physicochemical behavior of the enzyme as evidenced by measurements of sedimentation constant, reduced viscosity, and deuterium exchange.

Experimental Section

Preparation of Creatine Phosphokinase. The CPK employed in all of these studies was prepared according to the method of Mahowald *et al.* (1962) and had activities of 65–75 units/mg (Kuby *et al.*, 1954). The concentration of CPK was determined from the absorption at 280 mμ with the aid of the relation, $OD_{280m\mu} = 7.1 \times 10^4 c$ (moles/liter) (Noda *et al.*, 1954).

Inhibition by Iodoacetamide. The incubation mixture contained, if present (see Table I): Mg^{2+}, 5×10^{-3} M; CPK, 1.6×10^{-6} M (mol wt 81,000); iodoacetamide, 1×10^{-4} M; 0.17 M Tris–HCl buffer, pH 7.2; ATP or its analogs, 2×10^{-3} M; and creatine or its analogs, 2×10^{-2} M. Three milliliters of this reaction mixture was incubated for 15 min at 25°. A 0.2-ml aliquot was then removed for assay by the labile phosphate procedure of Kuby *et al.* (1954).

Inhibition by p-Nitrophenylacetate. The incubation mixture was the same as that described for iodoacetamide inhibition except that 2×10^{-3} M *p*-nitrophenylacetate replaced iodoacetamide. The extent of inhibition was evaluated after 30 min by removing a 0.2-ml aliquot for assay by the procedure of Kuby *et al.* (1954).

Trypsin Digestion of CPK. The reaction mixture contained, if present (see Table II): Mg^{2+}, 1.2×10^{-2} M; CPK, 2.13×10^{-5} M; ATP or other nucleotides, 1.5×10^{-3} M; creatine, 3×10^{-2} M; NaCl, 7.5×10^{-2} M; and glycine, 2.5×10^{-4} M. The total volume of the reaction mixture was 4 ml. Reaction was initiated by introducing 100 μl of 0.001 N HCl which contained 0.8 mg of crystalline trypsin (Worthington Biochemical Corp.). The pH was maintained at pH 9.0 with the aid of a Radiometer pH-Stat Titrigraph and the rate of digestion was determined from the rate of base consumption (Cunningham, 1964) between 3 and 6 min. The temperature was 30°.

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[1] Abbreviations used in this work: CPK, creatine phosphokinase; ATP, adenosine triphosphate; GDP, guanosine diphosphate; ADP, adenosine diphosphate.

TABLE I: Protection of CPK against Inhibition by Iodoacetamide and *p*-Nitrophenylacetate by Substrates and Substrate Analogs (pH 7.2, 25°).

Composition of the Reaction Mixture[a]		Inhibitor	
Creatine or Analog	ATP or Analog	Iodoacetamide (% of inhibition)	*p*-Nitrophenylacetate (% of inhibition)
−	−	44.0 ± 1.7	38.0 ± 3.0
+	+	7.6 ± 0.1	7.0 ± 2.0
+	−	43.9 ± 1.3	36.0 ± 1.0
−	+	43.9 ± 1.5	33.0 ± 1.0
−	ADP	40.6 ± 2.0	32.3 ± 1.5
+	ADP	13.0 ± 0.5	9.0 ± 2.0
+	GDP	43.1 ± 0.1	
+	dATP	24.0 ± 0.7	27.0 ± 1.5
+	AMP	42[b]	28.0 ± 1.4
+	GTP		35.0 ± 1.4
+	Adenine		33[b]
+	PRPP		36[b]
Creatinine	+	42.0 ± 0.7	26.0 ± 0.5
Glycocyamine	+	42[b]	23.0 ± 4.0
Guanidine	+	43[b]	28.0 ± 2.0
Taurocyamine	+		27.0 ± 1.8

[a] For concentration and complete details see the text. All samples contained CPK and Mg^{2+}. [b] These experiments were performed only once.

Preparation of Carboxymethylated CPK. The reaction mixture contained 1.11×10^{-4} M CPK, 9.37×10^{-3} M iodoacetamide, and 0.17 M Tris–HCl, pH 7.25. After 30-min incubation at 25°, 98% of the original activity was lost. Then the incubation mixture was dialyzed against 0.001 M glycine buffer (pH 9.0) at 4° for 17 hr.

Ultracentrifugal Analyses. Sedimentation velocity measurements were carried out in a Beckman Model E instrument at a speed of 59,000 rpm and temperatures near 20°. The sedimentation constants reported here were performed at two CPK concentrations (see Table III) and have been corrected to $s_{20,w}$. The solutions also contained: Tris–HCl buffer, 0.117 M, pH 7.2; Mg^{2+}, 0.025 M; creatine, 0.061 M; and, if present, ATP or other nucleotides, 5.82×10^{-3} M.

Viscosity Studies. Measurements were performed with a Cannon-Ubbelohde semimicro viscometer in a constant temperature bath at 30 ± 0.02°. The compositions of the solutions were the same as those described under ultracentrifugal analyses except that different concentrations (see Table IV) of CPK were present.

Deuterium Exchange. Measurements of the rate of exchange of D_2O with creatine phosphokinase were carried out by the infrared absorption technique as described by Hvidt (1963) and by Di Sabato and Ottesen (1965). The spectra were obtained with a Perkin-Elmer Model 12 ratio recording infrared spectrophotometer, and were made at a constant I_0 setting. Difference spectra of the protein in buffered D_2O *vs.* buffered D_2O in calcium fluoride cells of 0.1-mm length were made over the region 1760–1370 cm^{-1} at various time intervals after mixing up to about 4 hr. All runs were made in a constant temperature room at 21 ± 1°, and the cells were removed from the light path except when spectra were being recorded. The decrease in the absorption at 1552 cm^{-1}, the amide II band, was assumed to measure the rate of exchange of the amide hydrogens of the protein with deuterium of the solvent. The absorbancy at 1658 cm^{-1}, the amide I band, 0.44 for 1.86×10^{-4} M CPK, was used to normalize runs made at slightly different protein concentrations. The extinction coefficient per peptide bond at 1658 cm^{-1} was calculated to be 316 l. $mole^{-1}$ cm^{-1} (mol wt 81,000 and 735 amide hydrogens/mole (Noltmann *et al.*, 1962)), which may be compared with the values of 315 l. $mole^{-1}$ cm^{-1} and 353 l. $mole^{-1}$ cm^{-1} reported for heart lactic dehydrogenase (Di Sabato and Ottesen, 1965) and lysozyme (Hvidt, 1963), respectively. The background absorbancy at 1552 cm^{-1} was determined from a spectrum of the enzyme which had been heated 90 min at 80° in buffered D_2O containing 0.15 M sodium dodecyl sulfate. It was assumed that all amide hydrogens had exchanged after this treatment and the background absorbancy of the solution, 0.07 at a protein concentration of 15.1 mg ml^{-1}, is near that of similar preparations of the other enzyme noted previously (Hvidt, 1963; Di Sabato and Ottesen, 1965). The assumption was made (Hvidt, 1963; Di Sabato and Ottesen, 1965) that the absorbancy ratio before any exchange had occurred, amide II/amide I, was 0.46 after correction for background absorbancy at both frequencies. Detailed descriptions of this procedure have been pub-

TABLE II: The Rate of Trypsin-Catalyzed Hydrolysis of Creatine Phosphokinase in the Presence of Substrates and Substrate Analogs (pH 9.0, 30°).

Composition of Reaction Mixture[a]						Rate of Hydrolysis[c]
CPK	Mg^{2+}	Creatine	ATP	ADP	GDP	
+	+	−	−	−	−	1.00[b]
+	+	+	−	−	−	0.87 ± 0.05
+	+	−	+	−	−	0.88 ± 0.07
+	+	+	+	−	−	0.48 ± 0.05
+	+	−	−	+	−	0.70 ± 0.06
+	+	+	−	+	−	0.49 ± 0.04
+	+	+	−	−	+	0.82 ± 0.03
CPK (heat denatured)	+	−	−	−	−	25.2 ± 0.1[d]

[a] See text for concentrations. [b] Actual value: 0.13 μmole bonds hydrolyzed/min. [c] Average and maximum deviation of four experiments. [d] Initial rate of hydrolysis.

lished previously (Hvidt, 1963; Di Sabato and Ottesen, 1965). All D_2O solutions contained 0.12 M Tris buffer, pH 7.2, and 0.025 M magnesium acetate. When present, the concentration of CPK was about 15 mg/ml (1.85×10^{-4} M), of ADP 0.006 M, and of creatine 0.06 M.

Results

The data of Table I are in agreement with earlier observations (Watts and Rabin, 1962; Watts, 1963) that neither substrate alone affords much if any protection of CPK against inhibition by iodoacetamide or *p*-nitrophenylacetate, while together they reduce by more than 80% the expected inhibition. Of equal interest is the observation that ADP can substitute for ATP in the presence of creatine and Mg^{2+} and provide almost as much protection. Some degree of protection is afforded when deoxy-ATP is present along with creatine, but much less protection is seen with the other analogs examined. It may be noted that protection against iodoacetamide inhibition by various substrate and analog combinations parallels closely that against *p*-nitrophenylacetate inhibition, with the exception that several analogs that are completely ineffective against the former show slight protection against the latter.

We have also examined the susceptibility of CPK to tryptic digestion under various conditions. Creatine phosphokinase is relatively resistant to trypsin-catalyzed hydrolysis so that rather high protease concentrations were used. After a brief and rapid initial reaction, proteolysis proceeds in a linear fashion for up to 15 min. We have used the slopes of this linear portion to compare the digestibility of CPK in the presence of some of the substrates and analogs tested in the inhibition analysis of Table I. The results are shown in Table II and Figure 1. In the presence of Mg^{2+} either creatine or ATP alone produces an appreciable decrease in the trypsin susceptibility of CPK. Together, however, they reduce the rate of digestion to about half the control value. ADP is somewhat more effective than ATP alone and is equally effective in combination with creatine. On the other hand, GDP, a nonsubstrate nucleotide, produces minimal protection, even in combination with creatine.

It had also been reported earlier that in the simultaneous presence of both substrates CPK exhibited changed optical rotatory parameters, and this, together with marked changes in the antigenic properties of the enzyme, had been taken to reflect a rather pronounced, substrate-dependent change in configuration (Samuels *et al.*, 1961; Samuels, 1961). In order to extend the characterization of the physicochemical changes resulting from this enzyme–substrate interaction we have carried out studies of the sedimentation velocity and reduced viscosity of CPK in the presence of various substrate combinations: creatine and Mg^{2+}; creatine, Mg^{2+}, and ATP; creatine, Mg^{2+}, and ADP; and creatine, Mg^{2+}, and GDP. The results are shown in Tables III and IV. It may be seen that the observed

TABLE III: The Sedimentation Constants of CPK under Various Conditions.

Composition of the Solution	$s_{20,w}$ Concentration of CPK in the Solution	
	3.7 mg/ml	6.8 mg/ml
CPK–creatine–Mg^{2+}	5.36	5.13
CPK–creatine–Mg^{2+}–ATP	5.50	5.32
CPK–creatine–Mg^{2+}–GDP	5.45	5.20
CPK–creatine–Mg^{2+}–ADP	5.52	5.30

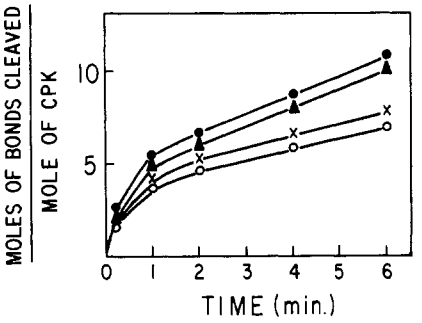

FIGURE 1: The tryptic digestion of CPK in the presence of various substrates and substrate analogs. For details, see the text: (—●—●—) CPK + Mg^{2+} + ATP; (—▲—▲—) CPK + GDP + creatine + Mg^{2+}; (—x—x—) CPK + ATP + creatine + Mg^{2+}; (—o—o—) CPK + ADP + creatine + Mg^{2+}.

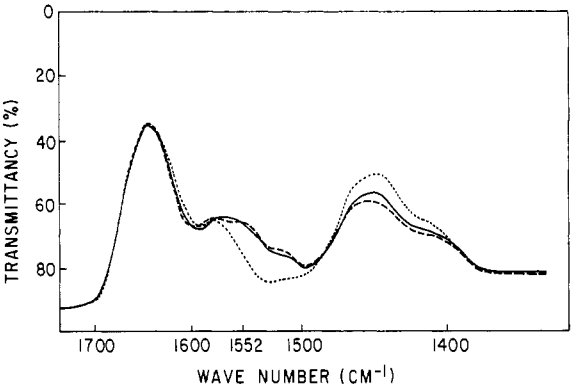

FIGURE 2: Infrared difference spectra in D_2O of creatine phosphokinase in Tris–HCl buffer at pH 7.1. Other additions: (———) Mg^{2+}; (— — —) Mg^{2+} + creatine + ADP; (- - - -) Mg^{2+} + sodium dodecyl sulfate, and heated 90 min at 70°. For concentrations and other details see the text. All spectra were begun approximately 4 min after solution of the protein in D_2O.

TABLE IV: The Reduced Viscosity of CPK under Various Conditions.

Composition of the Solution	Reduced Viscosity pH 7.2[a]	pH 9.0[b]
CPK–creatine–Mg^{2+}	0.0419 ± 0.0003	
CPK–creatine–Mg^{2+}–ATP	0.0394 ± 0.0003	
CPK–creatine–Mg^{2+}–GDP	0.0406 ± 0.0003	0.0408
CPK–creatine–Mg^{2+}–ADP	0.0389 ± 0.0003	0.0396

[a] The concentration of CPK used in these experiments was 0.79 g/100 ml. [b] These experiments were only performed once. The concentration of CPK employed here was 0.36 mg/100 ml; 0.1 M glycine buffer, pH 9.0 was used to substitute for the Tris buffer, pH 7.2.

maximum substrate-dependent change in $s_{20,w}$ does not exceed 4% and in (η_{sp}/C), about 5%. These differences, though systematic in the sense that ADP or ATP invariably produces a similar change in the constant measured with respect to that found in the absence of these nucleotides, are at the limit of experimental error of these procedures under our experimental conditions. They are perhaps best interpreted simply as indicating that any substrate-induced conformational change is relatively limited in magnitude.

The measurement of the rate and extent of exchange of the amide hydrogens of proteins for deuterium in buffered D_2O solutions has been shown by Linderstrøm-Lang (1955) and by others (Nielsen, 1960; Blout *et al.*, 1961; Hvidt, 1964; Di Sabato and Ottesen, 1965) to reflect to some extent the conformational stability of protein molecules. Thus at neutral pH, the amide hydrogens of simple di- and tripeptides exchange essentially instantaneously while amide hydrogens of polypeptides known to be in the α-helical conformation exchange only slowly. We have utilized the infrared absorption technique to measure the rate of decrease in the amide II band of CPK which in turn reflects the rate of deuterium exchange. Our results are illustrated in Figures 2 and 3. In Figure 2 are given typical spectra for CPK plus Mg^{2+} and CPK and ADP and creatine plus Mg^{2+}, both taken approximately 5 min after solution of the protein in D_2O containing the indicated substrates and Tris buffer. The small difference in the form of the spectra in the important 1540–1600 cm^{-1} region is of uncertain significance, but it may be seen that at 1552 cm^{-1}, the average position of the amide II band, the transmittancy of the two samples almost coincides. The spectrum of an exchanging solution containing CPK, ADP, and Mg^{2+} corresponds almost exactly with that of CPK plus Mg^{2+} alone. The other spectrum included in Figure 2 is that of a solution of CPK plus Mg^{2+} which also contained 0.15 M sodium dodecyl sulfate and which had been heated 90 min at 80°. All amide hydrogens in this denatured sample are presumed to have exchanged with D_2O. The similarity of these spectra to those of other proteins which have been investigated by this technique is marked (Hvidt, 1963; Hvidt *et al.*, 1963; Di Sabato and Ottesen, 1965). The rates of deuterium exchange, evaluated from a series of such spectra, are given in Figure 3. Between 60 and 65% of the amide hydrogens exchange instantaneously, while only 70 to 74% have exchanged after 4 hr. A small decrease in the rate of exchange could be detected when ADP was present, but the addition of creatine had no further effect upon the exchange. A comparison of the linear portion of the exchange curves indicates that ADP interaction with CPK slows the exchange of approximately 3–4% of the amide groups or 22–36 amide groups/mole.

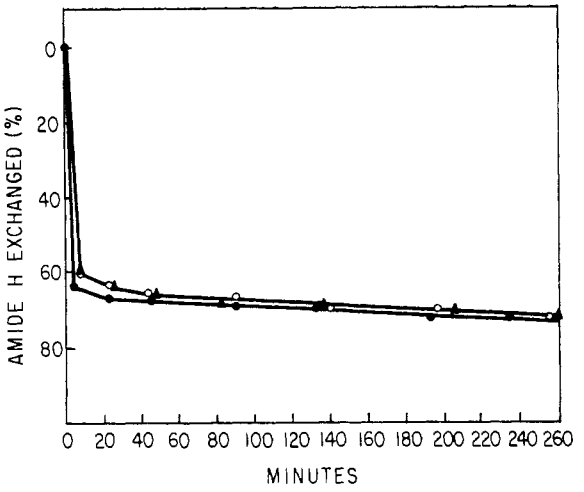

FIGURE 3: Rate of amide hydrogen exchange of creatine phosphokinase in the presence of different substrate combinations in Tris–HCl buffer at pH 7.1: (●) Mg^{2+} + creatine; (○) Mg^{2+} + ADP; (▲) Mg^{2+} + ADP + creatine. For concentrations and other details see the text.

Discussion

The limited reaction of iodoacetamide or of *p*-nitrophenylacetate with creatine phosphokinase produces an enzymatically inert protein. In each case the site of the reaction is an unusually reactive amino acid side chain, presumably in or near the active site of the protein. A considerable body of evidence (Watts *et al.*, 1961; Kuby *et al.*, 1962; Mahowald *et al.*, 1962; Thomson *et al.*, 1964) suggests that this enzyme has two essentially independent catalytic centers per molecule (mol wt 81,000), and it can be shown that the iodoacetate inhibition results from the conversion of two cysteine residues to *S*-carboxymethylcysteine (Mahowald *et al.*, 1962; Watts and Rabin, 1962). Inhibition by *p*-nitrophenylacetate has been less well defined but can be related to the acetylation of two identical but as yet unidentified residues (Clark and Cunningham, 1965). These reactions appear to be quite independent, since prior inhibition with iodoacetate does not affect the course of subsequent reaction with *p*-nitrophenylacetate (Clark and Cunningham, 1965). Watts and Rabin (1962) had previously pointed out that the rapidity and independence of pH of the iodoacetate reaction suggests an unusual reactivity of the sulfhydryl group of the protein. Similarly, acetylation of a protein by *p*-nitrophenylacetate at pH 7 requires a reaction site with properties different from those encountered in normal amino acid side chains (Clark and Cunningham, 1965). Although $MgATP^{2-}$ and $MgADP^{-}$ are bound rather strongly by CPK, $K_{diss} = 1 \times 10^{-4}$ and 6×10^{-5}, respectively, at pH 7.9 (Kuby *et al.*, 1962). Watts (1963) has shown that this interaction has at most a very small effect on the rate of the inhibitory reactions with iodoacetate and *p*-nitrophenylacetate. The presence of creatine likewise has little measurable effect on the inhibition reactions, but this is less informative since the dissociation constant reported (Kuby *et al.*, 1962) for creatine, $K_{diss} \geq 10^{-2}$, is much larger than those of the nucleotides.

The binding of $MgADP^{-}$ may however produce limited conformational alterations in the protein. We have been able to show that a small decrease in the number of amide hydrogens which can exchange with deuterium occurs when $MgADP^{-}$ is present either alone or together with creatine. This decrease corresponds to the involvement of from 22 to 36 amide groups, or about 11–18 per active site. It is interesting that Hvidt *et al.* (1963) estimated that 14 amide hydrogens per binding site of yeast alcohol dehydrogenase were prevented from exchanging in the presence of diphosphopyridine nucleotide. Similarly, the exchange of between 8 and 25 amide hydrogens per active site in chicken heart lactic dehydrogenase is slowed in the presence of diphosphopyridine nucleotide and reduced diphosphopyridine nucleotide (Di Sabato and Ottesen, 1965). Thus despite some uncertainty in the absolute significance of the number of exchangeable groups involved and the mechanism by which they are stabilized toward deuterium exchange (Hvidt, 1963; Di Sabato and Ottesen, 1965), it seems reasonable to conclude that the conformational effect of nucleotide binding in CPK is comparable to that observed with diphosphopyridine nucleotide in other proteins and does not exhibit any especially unusual characteristics. Kägi and Li (1965) have recently reported that $MgADP^{-}$ or $MgATP^{2-}$ binding to CPK results in the generation of an extrinsic Cotton effect, but were unable to detect any change in optical rotation which could be related to a conformational change in the protein.

The simultaneous addition of $MgADP^{-}$ and creatine does not produce any change in the rate or extent of deuterium exchange or in the optical rotatory properties of CPK (Kägi and Li, 1965) beyond those induced by $MgADP^{-}$ binding alone. This combination of substrates does however produce a marked protection against iodoacetamide and *p*-nitrophenylacetate inhibition. Watts had previously described this protective action of the MgATP–creatine combination, and had ascribed it to a special "working" enzyme conformation which included a nucleotide–creatine interaction through the exchangeable phosphate residue. The present observation of the effectiveness of the catalytically inactive substrate pair, MgADP–creatine, in preventing inhibition would seem to point to some other type of cooperative interaction as the basis of protection, and the lack of further changes in optical rotatory dispersion and deuterium exchange strongly imply that only very limited protein conformational changes can accompany the cooperative interaction of the substrates. Our failure to detect significant changes in $s_{20,w}$ and η_{sp}/C for CPK in the presence of $MgADP^{-}$ and creatine also limits the magnitude of possible conformational change and rules out any relationship to dissociation of this "divalent" enzyme into subunits.

The occurrence of a highly specific cooperative substrate interaction is further indicated by our measure-

ments of the tryptic susceptibility of CPK alone and in the presence of various substrate combinations. In the presence of $MgATP^{2-}$ or of creatine alone the enzyme undergoes a slow proteolysis by trypsin. The rate of proteolysis can be reduced by an amount exceeding the additive effects of each alone if a combination of these substrates is used. $MgADP^{-}$ is equally effective as $MgATP^{2-}$ in combination with creatine, while $MgGDP^{-}$, an analog known not to be a substrate of the enzyme, is completely ineffective. Tryptic susceptibility, of course, implies availability of lysine or arginine residues in the peptide chain, but the quantitative significance of such measurements in terms of conformational stability is not clear, especially in view of the other criteria which suggest minimal conformational changes throughout. Elödi and Szabolcsi (1959) have employed similar studies of tryptic susceptibility to follow substrate-induced conformational changes in glyceraldehyde 3-phosphate dehydrogenase.

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References

Blout, E. R., De Lozé, C., and Asadourian, A. (1961), *J. Am. Chem. Soc. 83*, 1895.

Clark, J. R., and Cunningham, L. W., (1965), *Biochemistry 4*, 2637.

Clark, J. R., Jacobs, H. G., and Cunningham, L. W. (1964), *Federation Proc. 23*, 423.

Cunningham, L. W. (1964), Hoppe-Seyler/Thierfelder Handbuch, Vol. VI A, Springer-Verlag, Berlin, p. 279.

Di Sabato, G., and Ottesen, M. (1965), *Biochemistry 4*, 422.

Elödi, P., and Szabolcsi, G. (1959), *Nature 184*, 56.

Hvidt, A. (1963), *Compt. Rend. Trav. Lab. Carlsberg 33*, 475.

Hvidt, A. (1964), *Compt. Rend. Trav. Lab. Carlsberg 34*, 299.

Hvidt, A., Kägi, J. H. R., and Ottesen, M. (1963), *Biochim. Biophys. Acta 75*, 290.

Kägi, J. H. R., and Li, T. K. (1965), *Federation Proc. 24*, 285.

Kuby, S. A., Mahowald, T. A., and Noltmann, E. A. (1962), *Biochemistry 1*, 748.

Kuby, S. A., Noda, L., and Lardy, H. A. (1954), *J. Biol. Chem. 209*, 191.

Linderstrøm-Lang, K. (1955), Special Publication No. 2, The Chemical Society, London, p. 1.

Mahowald, T. A., Noltmann, E. A., and Kuby, S. A. (1962), *J. Biol. Chem. 237*, 1535.

Nielsen, S. O. (1960), *Biochim. Biophys. Acta 37*, 146.

Noda, L., Kuby, S. A., and Lardy, H. A. (1954), *J. Biol. Chem. 209*, 203.

Noltmann, E. A., Mahowald, T. A., and Kuby, S. A. (1962), *J. Biol. Chem. 237*, 1146.

Samuels, A. J. (1961), *Biophys. J. 1*, 437.

Samuels, A. J., Nihei, T., and Noda, L. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1992.

Thomson, A. R., Eveleigh, J. W., and Miles, B. J. (1964), *Nature 203*, 267.

Watts, D. C. (1963), *Biochem. J. 89*, 220.

Watts, D. C., and Rabin, B. R. (1962), *Biochem. J. 85*, 507.

Watts, D. C., Rabin, B. R., and Crook, E. M. (1961), *Biochim. Biophys. Acta 48*, 380.