Conformative Response of Xanthosine 5′-Phosphate Aminase*

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ABSTRACT: Xanthosine 5′-phosphate aminase undergoes conformational change in response to certain of its substrates and closely related substrate analogs. This conformative response has been quantitated in terms of the molar concentration of xanthosine 5′-phosphate which causes a half-maximal response, the conformative response constant, or K_{cr}. The conformative response is detected as altered susceptibility to thermal inactivation and to digestion by proteases of different specificities. The measurement of K_{cr} by each of these methods yields identical values, 0.1 mM xanthosine 5′-phosphate. Adenosine triphosphate does not cause a conformative response, but it greatly modifies the response to xanthosine 5′-phosphate as shown by a 2200-fold decrease in K_{cr}. Adenosine triphosphate can be effectively replaced by a combination of adenosine and inorganic pyrophosphate as a modifier but not as substrate. The only both effective and analogous replacement for xanthosine 5′-phosphate is 2′-deoxyxanthosine 5′-phosphate which is also a substrate for the aminase. The other substrate, ammonia, has no effect on the response to xanthosine 5′-phosphate. However, ammonia eliminates modification of the conformative response by adenosine triphosphate but not by the adenosine and inorganic pyrophosphate combination. This suggests a key role for the catalytic intermediate, an adenylated xanthosine 5′-phosphate, which is produced on exposure of the aminase to adenosine triphosphate and xanthosine 5′-phosphate. According to alternative models the intermediate either stabilizes the modified response to xanthosine 5′-phosphate and adenosine triphosphate or itself is the inducer of the modified response. In either case aminolysis of the intermediate by added ammonia would eliminate the modification. The adenosine, inorganic pyrophosphate, and xanthosine 5′-phosphate combination is not subject to aminolysis; therefore, ammonia cannot eliminate the modified response to the combination. According to the first model adenosine and inorganic pyrophosphate serve as a replacement for adenosine triphosphate, whereas in the second model they are pictured as a substitute for the adenyl portion of the catalytic intermediate.

The interaction of an enzyme with substrates, substrate analogs, or other effectors is frequently accompanied by a readily detectable change in the conformation of the enzyme molecule. The term "conformative response" has been suggested (Citri and Zyk, 1967) to distinguish such change in conformation from generalized and nonspecific conformational modifications caused by a change in environment which does not selectively involve the stereospecific sites of the enzyme. A parameter which can be conveniently used for quantitative expression of the conformative response is the "conformative response constant," K_{cr}, which has been defined as the molar concentration of the substrate or other effectors which causes a half-maximal conformative response (Zyk and Citri, 1967).

In the present report we examine the conformative response of XMP aminase (xanthosine 5′-phosphate:ammonia ligase (AMP), EC 6.3.4.1.) to its substrates and its substrate analogs. The conformative response to XMP is reflected in resistance to inactivation by heat and proteolytic enzymes. The K_{cr} values for XMP obtained by three independent criteria are in excellent agreement. The other substrates, ATP and ammonia, cause no conformative response by any of these criteria. The K_{cr} value for XMP is, however, drastically modified by the other substrates as well as by the inhibitors. A study of the effect of these modifiers reveals a pattern of responsiveness by XMP aminase which may explain several aspects of its catalytic behavior.

Materials and Methods

Chemicals. d-XMP was prepared by deamination of d-GMP with HNO_2. Other purine nucleotides and nucleosides, trypsin, and Pronase were commercial preparations.

Purification of XMP Aminase. A previously published purification procedure was employed (Fukuyama and Moyed, 1964). There were modifications in the strain of bacteria and in the method of cultivation. The bacterial strain was *Escherichia coli* B-96, a purine-requiring mutant blocked in inosinicase. Derepressed synthesis of XMP aminase was achieved in strain B-96 by growth for 8 hr in a mineral salts–glucose medium supplemented with 2 mg/ml of Bacto Difco Casamino Acids (vitamin free) and 40 μg/ml of AMP.

XMP Aminase Assay. XMP aminase was measured by following the decrease in absorption at 290 mμ at pH 8.5 due to the conversion of XMP into GMP. The reagent mixture contained 180 μmoles of Tris-HCl (pH 8.5), 25 μmoles of $MgCl_2$, 3 μmoles of ATP, 0.3 μmole of XMP, and 160 μmoles of $(NH_4)_2SO_4$ in a total volume of 2.2 ml. The reaction was started by adding 0.3 ml of the enzyme preparation (3–10 μg of protein). The assay temperature was 39°.

Heat Treatment. XMP aminase (4–7 μg of protein), 1.5 μmoles of Tris-HCl (pH 7.4), 2.49 μmoles of $MgCl_2$, and sub-

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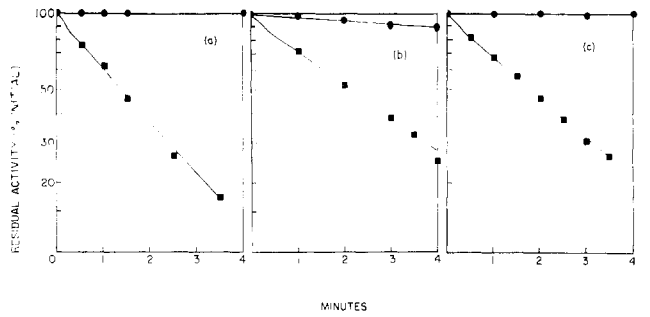

FIGURE 1: The effect of XMP on the rate of inactivation of XMP aminase. XMP aminase (6.5 μg of protein) was incubated with (●) and without (■) 1.0 mM XMP. The methods of inactivation, heat treatment (a), digestion by trypsin (b), and digestion with Pronase (c) are described in the text.

strates or modifiers in a total volume of 0.30 ml were incubated in a water bath at 55°. The heat treatment was terminated by dilution with 2.2 ml of the assay reagents kept at 39°. The mixture was then transferred to cuvets for assay of the residual activity.

Pronase Treatment. Stock solutions of Pronase (1.0 mg/ml of 10 mM $CaCl_2$) were stored at −17° and freshly diluted for each experiment. XMP aminase (5–10 μg of protein), 1.5 μmoles of Tris-HCl (pH 7.4), 2.49 μmoles of $MgCl_2$, 2 μg of Pronase, and substrates or modifiers in a volume of 0.30 ml were incubated at 37° in a water bath for the required time intervals. The residual activity was assayed following transfer of the mixture to cuvets.

Trypsin Treatment. Stock solutions of trypsin (10 mg/ml of 25 mM $CaCl_2$) were stored at −17° and diluted prior to each experiment. The procedure was otherwise identical with that employed for the treatment with pronase as described above.

Results

Inactivation of XMP Aminase by Heat and by Proteolytic Enzymes. XMP aminase is inactivated by heating at 55° or by proteolysis with either trypsin, papain, or Pronase. The initial rates of the thermal and proteolytic inactivation follow first-order kinetics with respect to XMP aminase (Figure 1). One of the substrates, XMP, when present in excess (1 mM) completely prevents inactivation by heat and by Pronase; similarly inactivation by trypsin is greatly reduced but still perceptible even in the presence of an excess of XMP (Figure 1). On first view these observations suggest that XMP aminase responds to XMP by a change in its conformation which is detectable by the increased stability to heat and proteolysis. Furthermore, it seems possible that each of the three treatments illustrated in Figure 1 can serve to quantitate the conformative response to XMP provided the assumption is correct that a change in the rate of inactivation by any one of the three treatments reflects a single, XMP-induced change in the conformation of the enzyme. For validity this assumption requires the proportion of the enzyme which is stabilized by XMP to be a function of the concentration of this substrate and to be independent of the criteria used for testing stability. Evidence that these requirements are met is presented in the following section.

Determination of F_{cr} Values for XMP Aminase. The calculation of F_{cr} values from the effects of a ligand on the rate of inactivation of an enzyme is based on the following considerations. If the conformative response to a ligand alters the stability of the enzyme, the fraction of the enzyme which has responded is defined as

$$F_{cr} = \frac{(E')}{(E_t)} = \frac{(E_t) - (E)}{(E_t)} = 1 - \frac{(E)}{(E_t)} \quad (1)$$

where (E′) is the concentration of the enzyme in the altered conformation, (E) is the concentration of the native enzyme, and (E_t) is the total concentration of the enzyme.

Since the rate of inactivation of E′ differs from that of E, the observed rate of inactivation in the presence of a ligand is

$$V = k_1(E') + k_2(E) \quad (2)$$

where k_1 and k_2 are the pseudo-first-order rate constants for the inactivation of the enzyme in the altered and native conformations, respectively. In the absence of the ligand the observed rate of inactivation is

$$V_0 = k_2(E_t) \quad (3)$$

whereas with saturating ligand (E′) = (E_t); thus the observed rate is

$$V_s = k_1(E_t) \quad (4)$$

TABLE I: Effect of ATP and XMP on the Rate of Inactivation of XMP Aminase.

Treatment[a]	Addn (0.1 mM)	V/V_0[b]	R[c]	F_{cr}[d]
Heat	ATP	0.96 ± 0.05 (4)		0
	XMP	0.49 ± 0.08 (4)	0.01	0.51
	ATP + XMP	0.09 ± 0.02 (3)	0.01	0.91
Pronase	ATP	0.98 ± 0.03 (3)		0
	XMP	0.49 ± 0.03 (3)	0.01	0.51
	ATP + XMP	0.00 ± 0.01 (3)	0.01	1.00
Trypsin	ATP	1.06 ± 0.07 (4)		0
	XMP	0.50 ± 0.02 (3)	0.04	0.52
	ATP + XMP	0.03 ± 0.04 (6)	0.04	1.00

[a] XMP aminase (6.5 μg of protein) was treated with Pronase, trypsin, and heat as described in the text but with the indicated additions. [b] The rates of inactivation in the presence of substrates, V, and in the absence of substrates, V_0, were calculated from the linear slopes obtained by plotting the log of residual activity of XMP aminase against duration of treatment. Under these conditions the rates of inactivation followed first-order kinetics over a period of 4 min. The ratios of V/V_0 listed are average values; the number of determinations is listed in brackets. [c] The values for R, (V_s/V_0), were obtained by extrapolating to infinite substrate concentrations. [d] The fraction of the enzyme which showed a conformative response, $F_{cr} = (1 - (V/V_0))/1 - R$, was derived as described in the text.

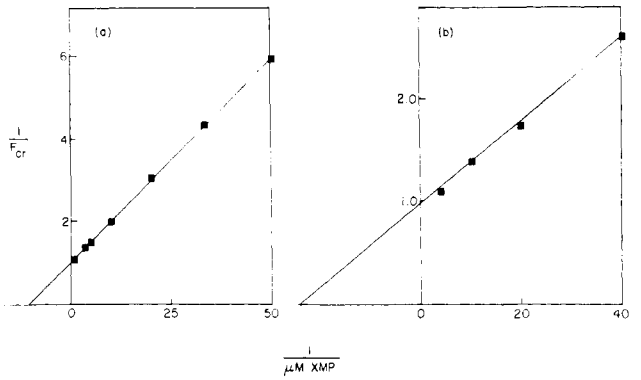

FIGURE 2: K_{cr} for XMP based on susceptibility to inactivation by Pronase. The aminase (6.5 μg of protein) was treated with Pronase as described in the text except that varying amounts of XMP (a) or varying amounts of XMP and 0.1 mM ATP (b) were added. Derivation and determination of F_{cr} and K_{cr} are described in the text. The K_{cr} values are (a) 100 μM XMP in the absence of ATP and (b) 0.045 μM XMP in the presence of 0.1 mM ATP.

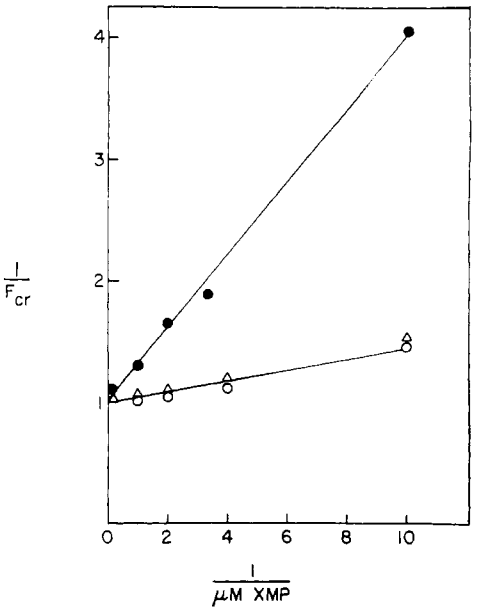

FIGURE 3: Comparison of the effect of ATP on K_{cr} values derived from the rates of thermal and proteolytic inactivation of XMP aminase. The aminase (6.5 μg of protein) was treated with Pronase (○), trypsin (▲), and heat (●), as described in the text except that 0.1 mM ATP and varying amounts of XMP were added. The K_{cr} values derived from the respective slopes are 0.045 μM for both tyrpsin and Pronase treatments and 0.30 μM for treatment with heat.

The ratio, R, is thus defined as

$$R = \frac{V_s}{V_0} = \frac{k_1}{k_2} \tag{5}$$

Dividing eq 2 by eq 3 and substituting R for k_1/k_2 gives

$$\frac{V}{V_0} = R\frac{(E')}{(E_t)} + \frac{(E)}{(E_t)} \tag{6}$$

Substitution of $(E)/(E_t)$ from eq 6 into eq 1 and rearrangement yields

$$F_{cr} = \frac{1 - V/V_0}{1 - R} \tag{7}$$

The experimental data required for the determination of the fraction of an enzyme, F_{cr}, which responds to the presence of a given concentration of a ligand are indicated by eq 7. In the experiments summarized in Table I we compared the F_{cr} values for 100 μM XMP using three independent criteria for the conformative response. The rates of inactivation of the aminase in the presence and in the absence of XMP are illustrated in Figure 1, and the corresponding values for V and V_0, respectively, were derived from the linear slopes obtained in such semilogarithmic plots. The R values were obtained by extrapolation. The ratios of V/V_0 were plotted against the reciprocals of the corresponding concentrations of XMP; the vertical intercept is V_s/V_0 or R. The F_{cr} values were calculated by substituting for V/V_0 and R in eq 7. The F_{cr} values for 100 μM XMP and the R values obtained by three methods of treatment, heat, Pronase, and trypsin, are listed in Table I. The values, based on several determinations, are in excellent agreement and indicate that the extent of stabilization is determined by the concentration of XMP, and is independent of the method of treatment.

Replacement of XMP by equimolar concentrations of another substrate, ATP, has no effect on the rate of inactivation of the enzyme by any of these criteria. However, ATP has a marked effect on the conformative response to XMP; the F_{cr} values in the presence of 0.1 mM XMP are doubled by the addition of 0.1 mM ATP which has no detectable effect by itself (Table I).

While the F_{cr} values obtained with XMP alone do not vary with the method of inactivation, there is a slight but experimentally significant, method-dependent difference in the F_{cr} values for a combination of XMP and ATP; the proportion of enzyme showing the conformative response is 91% as measured by thermal inactivation and 100% as measured by proteolytic inactivation (Table I). In addition to this difference, the effect of ATP raises another interesting point. Does the extensive stabilization observed in the presence of both substrates result from an increased binding of XMP caused by ATP itself or by the formation of the enzyme-bound intermediate, adenyl-XMP (Fukuyama, 1966)?

Conformative Response Constant, K_{cr}, for XMP. The concentration of the ligand which causes half-maximal conformative response, K_{cr}, has been estimated by a method analogous to that for the graphic determination of K_m (Citri and Zyk, 1967). The important difference between the two constants is that K_{cr} is calculated from data reflecting a change in enzyme conformation while K_m is calculated from changes in catalytic activity. Although both sets of data unquestionably emanate from the formation of an enzyme–ligand complex, it has been pointed out that assumption as to their identity may not be valid (Zyk and Citri, 1967).

In the present context the K_{cr} for XMP is operationally defined as the molar concentration of XMP required for half-maximal stabilization ($F_{cr} = 0.5$). The F_{cr} values for various XMP concentrations determined by three independent methods, inactivation by heat, Pronase, and trypsin, were

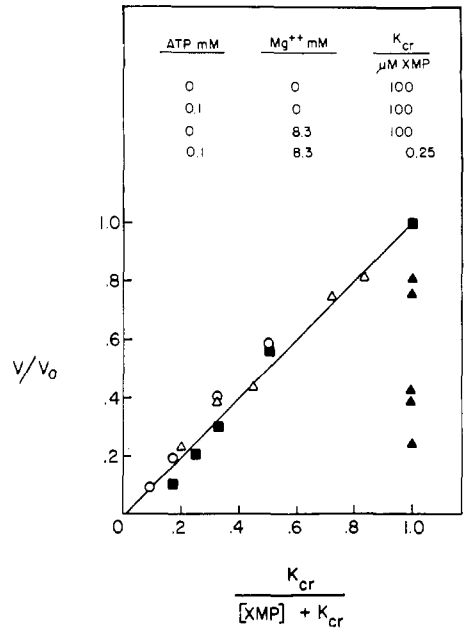

FIGURE 4: The effect of ATP on the conformative response. Requirement for Mg^{+2}. XMP aminase (6.5 μg of protein) was exposed to thermal inactivation as described in the text except that varying amounts of XMP were employed along with these additional variations: (○) no additions, 8.3 mM $MgCl_2$ present; 0.1 mM ATP, $MgCl_2$ omitted; and (△, ▲) 0.1 mM ATP and 8.3 mM $MgCl_2$. Rates of inactivation in the presence, V, and absence, V_0, of XMP and the F_{cr} values were determined as described in Figure 1 and text. The K_{cr} values, derived as in the Figure 2, are listed in the inset. The results obtained in the presence of both ATP and Mg^{2+} are plotted twice; (△) with K_{cr} = 0.25 μM as derived for this combination and (▲) with K_{cr} = 100 μM, as derived for the other combinations.

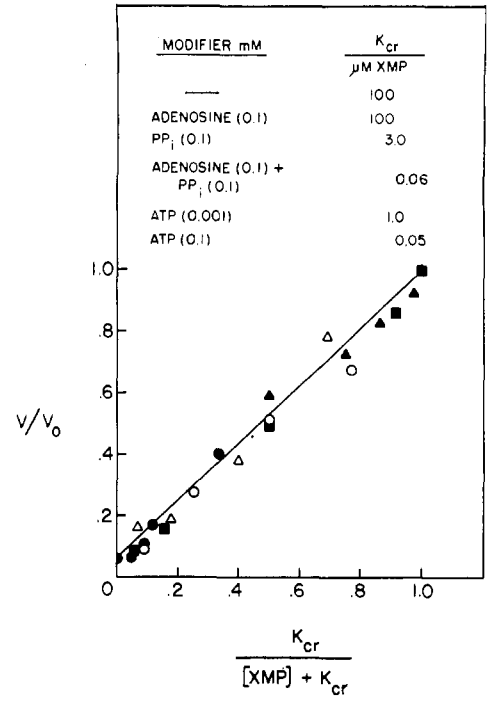

FIGURE 5: Replacement of ATP with structurally related modifiers of the conformative response. XMP aminase (6.5 μg of protein) was exposed to proteolytic inactivation by trypsin as described in the text except that varying amounts of XMP were employed along with these additional variations: (●) 0.1 mM ATP; (○) 0.001 mM ATP; (▲) 0.1 mM PP_i; (△) 0.1 mM adenosine and 0.1 mM PP_i; and (■) no additions or adenosine alone. Rates of inactivation in the presence, V, and absence, V_0, of XMP and the F_{cr} values were determined as described in Figure 1 and text. The K_{cr} values, derived as in Figure 2, are listed in the inset.

found to be in close agreement. Typical results obtained with Pronase are illustrated in Figure 2a. The reciprocals of the values were plotted against the reciprocals of the corresponding molar concentration of XMP, and the K_{cr} values were determined directly from the slope or from the horizontal intercept, which is equal to $-1/K_{cr}$. The K_{cr} value obtained by this procedure as well as by tryptic and thermal inactivation is equal to 100 μM XMP.

The effect of added ATP (0.1 mM) on the K_{cr} values for XMP is illustrated by the results obtained with Pronase (Figure 2b). The values of K_{cr} for XMP in the presence of ATP obtained by three different methods of treatment are compared in Figure 3. In each case the response to increasing concentrations of XMP is dramatically enhanced by the presence of ATP. The observed K_{cr} values based on the rates of proteolytic inactivation are identical, 0.045 μM XMP. Since the latter values were obtained at concentrations of enzyme representing a small but significant fraction of the concentrations of XMP employed, the correct K_{cr} values may be even lower. Thus, in the presence of ATP, which itself has no effect on the rate of proteolytic inactivation of XMP aminase, the concentration of XMP required for half-maximal protection is decreased by a factor of at least 2200. The K_{cr} value obtained from the rates of thermal inactivation is 0.30 μM XMP, lower by a factor of 330 than the value obtained in the absence of ATP.

Effect of ATP on the Conformative Response. REQUIREMENT FOR Mg^{2+}. Magnesium ions (Moyed and Magasanik, 1957) or other divalent metals are required for the catalytic reaction of XMP aminase. In the present experiments we examined the effect of Mg^{2+} on the conformative response to XMP and on its ATP-dependent modifications. The K_{cr} values were determined on the basis of proteolytic and thermal rates of inactivation as usual except that Mg^{2+} was omitted. The results are illustrated in Figure 4 where the V/V_0 ratios observed in a typical thermal inactivation experiment are plotted against the corresponding values of K_{cr}/(XMP) + K_{cr}. This plot was chosen for its convenience in presenting widely divergent K_{cr} values on a single set of coordinates. In this plot the slope is a theoretical straight line drawn between the point for (XMP) = 0 where $V = V_0$ and the point for (XMP) approaching infinity where $V = V_s$. The intercept in this plot is V_s/V_0 or R (eq 5) and depends upon the nature of the inactivating treatment (Table I). The experimental values expressed as V/V_0 and plotted as a function of K_{cr}/(XMP) + K_{cr} cluster along the slope provided the correct K_{cr} value is substituted in the abscissa. In the experiments summarized in Figure 4, the unmodified K_{cr} value for XMP, 100 μM, was not affected by the addition of Mg^{2+} or ATP alone; but when both ATP and Mg^{2+} were added the K_{cr} value was 0.25 μM XMP. It is clear that neither Mg^{2+} nor ATP alone modifies the conformative response to XMP.

The requirement for Mg^{2+} for modification of the conformative response to XMP was confirmed by results obtained with both trypsin and Pronase. In these experiments the re-

quirements for Mg^{2+} were even more pronounced since the modified K_{cr} value obtained by measurement of proteolytic inactivation, with either trypsin or Pronase was considerably lower (0.045 μM) than that obtained by thermal inactivation (0.25 μM).

Replacement of ATP as a Modifier of the Conformative Response. Results reported so far indicate that the requirement for enhancement of the conformative response to XMP, and thus presumably the binding of XMP, are the same as for the formation of the catalytic intermediate. However, previous studies on inhibition of the aminase by the adenine glycoside antibiotic, psicofuramine, and PP_i, revealed a considerable enhancement of XMP binding in the presence of psicofuranine and of PP_i (Fukuyama and Moyed, 1964). The similar requirement for PP_i and XMP for inhibition by adenosine suggested that the adenosine–PP_i combination might also cause an increase in XMP binding and thereby an enhancement of the conformative response to XMP under circumstances in which the catalytic intermediate could not be formed. Indeed, an equimolar, 0.1 mM, combination of adenosine and PP_i proved to be an excellent substitution for ATP in modifying the conformative response to XMP. The K_{cr} value obtained with this combination, 0.06 μM XMP, is very close to that obtained with ATP, 0.05 μM (Figure 5). Interestingly 0.1 mM adenosine by itself does not have an effect on the conformative response to XMP, whereas 0.1 mM PP_i by itself causes a 33-fold decrease in K_{cr}, an effect comparable with that observed with a much lower concentration of ATP, 0.001 mM (Figure 5).

The results described above were calculated from the rates of tryptic inactivation. Identical results were obtained with Pronase. For comparison with the experiments based on proteolysis a similar experiment was carried out with thermal inactivation (Figure 6). Although the results obtained by thermal inactivation differ quantitatively from the results based on proteolysis they lead to the same conclusion. As indicated before, the heat treatment appears to moderate the effects of ATP on the conformative response to XMP; the K_{cr} value in the presence of 0.01 mM ATP is decreased by a factor of 400 as determined by rates of thermal inactivation as compared with a reduction of 2200 in the proteolytic determinations (Figure 3). The replacement of ATP by the equimolar concentration of adenosine and PP_i causes precisely the same decrease in the K_{cr} value for XMP (Figure 6). The partial and proportionate modification caused by PP_i alone is also evident (Figure 6). In all cases Mg^{2+} was required for the modification of the conformative response (Figures 4 and 6).

Other replacements have been studied in terms of the following criteria. A compound replacing XMP should itself, like XMP, increase the stability of the enzyme to proteolytic or thermal inactivation; furthermore, its stabilizing effect should be enhanced by ATP or by adenosine and PP_i. The compound(s) replacing ATP should alone, like ATP, cause no increase in stability to either proteolytic or thermal inactivation, but such replacements for ATP should enhance stabilization by XMP. The replacement combinations which have been studied in terms of effects on rate constants of tryptic inactivation are listed in Table II. AMP[1] provides partial

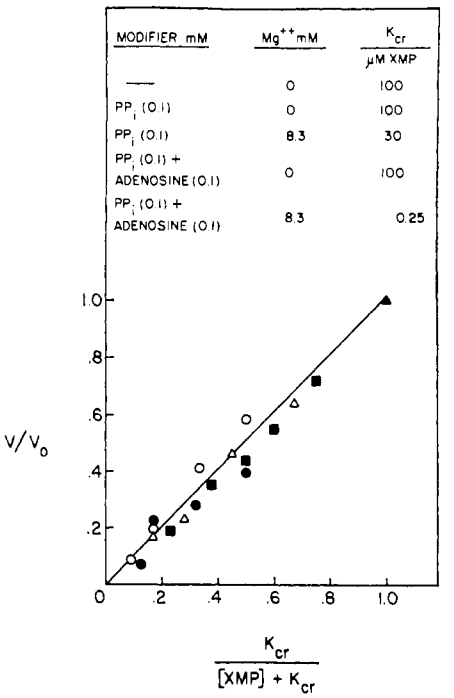

FIGURE 6: ATP-replacing modifiers. Requirement for Mg^{2+}. XMP aminase (6.5 μg of protein) was exposed to thermal inactivation as described in the text except that varying amounts of XMP were employed along with these additional variations: (●) 0.1 mM adenosine, 0.1 mM PP_i, $MgCl_2$ omitted; (■) 0.1 mM PP_i, 8.3 mM $MgCl_2$; (△) 0.1 mM adenosine, 0.1 mM PP_i, 8.3 mM $MgCl_2$; and (○) no other additions. For additional details, see Figure 5.

replacement of ATP as a modifier of the conformative response to XMP.

XMP can be replaced by dXMP. The stabilizing effect of dXMP is more pronounced in the presence of PP_i and greatly enhanced by adenosine–PP_i as well as by ATP (Table II). Thus the effect of dXMP is very similar to that of XMP.

The effect of GMP on the conformative response is not analogous to that of either XMP or ATP. GMP, like XMP, in the presence of ATP causes a conformative response as measured by tryptic inactivation; but unlike XMP, GMP has little effect alone or in combination with adenosine and PP_i (Table II). Comparing the effects of GMP with those of ATP it was found that GMP, like ATP, enhances the conformative response to XMP as measured by tryptic inactivation (Table II); but, unlike ATP, GMP by itself causes a conformative response as measured by thermal inactivation (Table III).

The specificity of XMP as an inducer of the conformative response was also examined by surveying the effects of two levels of structurally related compounds on the stability of the enzyme to thermal inactivation (Table III). Xanthosine and IMP had noticeable stabilizing effects only at the higher concentration, 1.0 mM. The only compound which was found to be both an effective and analogous replacement for XMP is the closely related analog, dXMP. Significantly, this compound is the only known analog of XMP which functions in

[1] Interpretation of effects of ADP are clouded by contamination of commercial preparations of this compound with 4 and 7% of AMP and ATP, respectively. Nevertheless, a labilizing effect of 0.1 mM ADP is probably attributable to ADP itself rather than to the contaminants as neither ATP nor AMP has such an effect. On the other hand, moderate enhancement of the conformative response to XMP observed with ADP could be readily accounted for by the contaminating ATP.

TABLE II: Effects of XMP, XMP Analogs, and Modifiers of the Conformative Response on the Rate of Inactivation of XMP Aminase.[a]

Substrate = 0.1 mM	Modifier =	0	0.1 mM	1.0 mM
			k'	
		0.37		
	PP_i		0.38	
	Adenosine		0.37	*b*
	AMP		0.36	0.34
XMP		0.19		
dXMP		0.26		
GMP		0.36		
XMP	PP_i		0.11	
d-XMP	PP_i		0.20	
d-XMP	PP_i + adenosine		0.04	
d-XMP	ATP		0.03	
GMP	PP_i		0.35	
GMP	PP_i + adenosine		0.35	
GMP	ATP		0.14	
XMP	Adenosine		0.37	*b*
XMP	AMP		0.16	0.03
XMP	ATP		0.03	
XMP	GMP		0.15	0.09
XMP	PP_i + adenosine		0.01	*b*

[a] Pseudo-first-order rate constants, k', for tryptic inactivation were determined from the linear slopes obtained by plotting the log of residual activity against time. XMP aminase (6.5 μg of protein) was treated with trypsin as described in the text except that additions indicated above were made. [b] With these additions even after dilution for assay of residual activity the aminase was completely inhibited.

TABLE III: Effect of XMP Analogs on the Rate of Thermal Inactivation of XMP Aminase.[a]

Compound	0.1 mM	k'	1.0 mM
		0.59	
Xanthosine	0.58		0.51
IMP	0.59		0.49
GMP	0.50		0.28
dXMP	0.45		0.22
XMP	0.29		0

[a] Pseudo-first-order rate constants, k', for thermal inactivation were determined from the linear slopes obtained by plotting the log of residual activity against duration of treatment. XMP aminase (6.5 μg of protein) was heated at 55° as described in the text except that the additions indicated above were made.

the catalytic reaction; it is probably also significant that dXMP, which is less potent than XMP as an inducer of the conformative response (Tables II and III), is also less effective as a substrate, yielding a tenfold higher K_m value in the catalytic reaction than XMP (H. S. Moyed, 1962, unpublished data).

Effect of Ammonia and Other Electrolytes on Stability and Conformative Response. Study of the effect of the third substrate, ammonia, on the conformative response of the aminase presented several problems. In the conventional catalytic reaction ammonia is supplied in the form of an ammonium salt. Thus, the effective concentration of this substrate is determined by the pH of the media; and even at the relatively high pH of the reaction, pH 8.5, a very high concentration, 80 mM, of the ammonium salt is required to maintain near-saturating concentrations of the substrate, ammonia (Moyed and Magasanik, 1957). The attempt to dissociate the nonspecific salt effects and the effects of pH from the stereospecific effect of ammonia is summarized in Table IV. The results are presented in terms of rate constants of thermal inactivation. Essentially similar results were obtained in proteolytic inactivation studies, but their interpretation was further complicated by the effect of salt concentrations and pH changes on the catalytic properties of the proteases. The results obtained from thermal inactivation (Table IV) indicate a general stabilizing effect of electrolytes at both of the pH values employed. The ammonium salt has no significant effect on stability in the presence of XMP, adenosine, and PP_i. In contrast, the enhancing effect of ATP on the XMP-induced stabilization is specifically eliminated by ammonium salts at pH 8.5, but not at pH 7.4. Thus, aminolysis of the catalytic intermediate appears to eliminate the ATP-dependent modification of the conformative response to XMP. There is no indication of a direct effect of ammonia on the conformation of the enzyme.

Comparison of K_{cr} and K_m Values. The unmodified and variously modified K_{cr} values for XMP were compared with the K_m value for XMP, 34 μM, which was determined in the usual way (Table V). The ratio of K_m:K_{cr} varies from 0.3 to 760. A discrepancy between the K_m and K_{cr} values was not unexpected since one of the substrates, ATP, acts as a powerful modifier of the conformative response while another substrate, ammonia, counteracts the modification to a considerable extent. The significance of this discrepancy will be considered in further detail in the discussion.

Discussion

A change in susceptibility to proteolysis often provides a sensitive indication of the change in the conformation on a protein. It is also frequently observed that enzymes are stabilized against proteolysis by substrates and other stereospecific ligands (Adelman *et al.*, 1968; Jacobs and Cunningham, 1968; Lui and Cunningham, 1966; Rupley, 1967; Trayser and Colowick, 1961). Thus it seems logical to conclude that the binding of such ligands causes a change in the conformation which is reflected in stabilization toward proteolysis, provided that the alternative explanations are reasonably excluded. While nonspecific effects, such as inhibition of the proteolytic activity by the ligand, can be readily evaluated, the possibility that the ligand protects the enzyme simply by masking a critical bond is difficult to rule out. It is rendered rather unlikely, however, if inactivation by proteolytic en-

TABLE IV: Effect of Ammonia and Electrolytes on the Rate of Thermal Inactivation of XMP Aminase.[a]

XMP	Modifiers	Other Addn (50 mM)	NH_2 (mM) pH 7.4	NH_2 (mM) pH 8.5	k' pH 7.4	k' pH 8.5
					0.51	0.74
		NaCl			0.42	0.41
		NH_4Cl	0.7	8.9	0.51	0.54
		$(NH_4)_2SO_4$	1.4	17.8	0.43	0.41
0.01					0.46	0.67
0.01	Adenosine, PP_i				0.09	0.07
0.01	Adenosine, PP_i	NaCl			0.08	0.07
0.01	Adenosine, PP_i	NH_4Cl	0.7	8.9	0.11	0.07
0.01	Adenosine, PP_i	$(NH_4)_2SO_4$	1.4	17.8	0.08	0.07
0.10					0.29	0.35
0.10	ATP				0.05	0.06
0.10	ATP	NaCl			0.03	0.07
0.10	ATP	NH_3Cl	0.7	8.9	0.08	0.34
0.10	ATP	Na_2SO_4			0.05	0.06
0.10	ATP	$(NH_4)_2SO_4$	1.4	17.8	0.11	0.32

[a] The procedures were the same as in Table III, except where the standard buffer was replaced by pH 8.5 Tris buffer (5 mM).

zymes of different specificity is retarded to the same extent by the presence of the ligand. In the present case we use trypsin and Pronase and an additional, independent criterion, thermostability, which is not subject to similar reservations. The excellent agreement between the values obtained by the three criteria has led us to assume that each procedure yields valid information on the conformative response of the enzyme to XMP. Conclusive evidence for a conformational transition depended, however, upon demonstrating that the binding of XMP modifies the reactivity of other stereospecific sites in the enzyme. Such evidence has now been obtained for the XMP-dependent interaction of XMP aminase with pyrophosphate and adenosine. The previous observation (Fukuyama and Moyed, 1964) that the binding by XMP aminase of the adenine-glycoside antibiotic, psicofuranine, depends upon the presence of XMP and pyrophosphate is undoubtedly related to a similar conformational transition (N. Zyk, N. Citri, and H. S. Moyed, 1968, unpublished data).

The present quantitative treatment of the rates of inactivation as a function of XMP concentration requires one further comment. It is commonly assumed that the concentration of the substrate required for half-maximal protection (K_{cr} in the present terminology) is the same as that required for half-saturation of the enzyme. This assumption is implicit in the derivation of dissociation constants for an enzyme–substrate complex from measurements of substrate-dependent changes in the stability or reactivity of the enzyme (Trayser and Colowick, 1961; Jacobs and Cunningham, 1968; Adelman *et al.*, 1968; O'Sullivan *et al.*, 1966; O'Sullivan and Cohn, 1966). Such an assumption is not necessarily warranted, since it implies that the initiation and termination of the conformative response coincide precisely with the formation and dissociation of the enzyme–substrate complex (Zyk and Citri, 1967, 1968). The relation between the K_{cr} values obtained here and dissociation constants based on direct binding studies will be examined in a subsequent report.

The effect of ATP and the antagonistic effect of ammonia, as reflected by modification of the conformative response, are almost certainly related to the catalytic function of the enzyme. Several models could account for these relationships. According to one model the binding of XMP results in a primary response (characterized by $K_{cr} = 100\ \mu M$) promoting the binding of ATP, which in turn results in a secondary response ($K_{cr} = 0.05\ \mu M$); the secondary response is required for the

TABLE V: Summary of Direct and Modified K_{cr} Values for XMP.

Modifier (mM)	Method[a]	K_{cr}[b] (μM)	K_m[c]:K_{cr}
	H	100	0.3
	P	100	0.3
	T	100	0.3
PP_i (0.1)	H	30	1
PP_i (0.1)	T	3	11
PP_i (0.1) + adenosine (0.1)	H	0.20	170
PP_i (0.1) + adenosine (0.1)	T	0.06	570
ATP (0.001)	T	1.0	34
ATP (0.1)	H	0.28	120
ATP (0.1)	P	0.045	760
ATP (0.1)	T	0.045	760

[a] Thermal inactivation (H), digestion by Pronase (P), and digestion by trypsin (T) are described in the text. [b] Determination of K_{cr} values is described in Figure 2. [c] The assay procedure described in the section on Materials and Methods but with varying, rate-limiting amounts of XMP were used for the determination of the Michaelis constant, K_m, which was found to be 0.34 mM XMP.

formation of the catalytic intermediate, adenyl-XMP; aminolysis of the intermediate is followed by relaxation of the conformative response. According to such a model the cycle is arrested at the stage of the primary response in the presence of XMP alone or at the stage of the secondary response when ATP and Mg^{2+} are added. A corollary of this scheme is that the formation of an intermediate is not required for the modification of the conformative response. In fact, it is possible to experimentally replace ATP by compounds bearing sufficient resemblance to relevant components of this substrate. In terms of the above scheme, the cycle may be arrested at various stages intermediate between the primary and the secondary response, depending upon how effective the replacement is. (For specific examples, see Table II.) In the case of adenosine and PP_i, the replacement combination was accepted by the enzyme as equivalent to ATP in terms of modifying the conformative response, even though these compounds cannot interact with XMP.

Another possibility, namely, that the enzyme accepts the combination of XMP–adenosine–PP_i as equivalent to the catalytic intermediate, suggests an alternative model which is considerably simpler. According to that model a single conformative response is induced by a relatively high concentration of XMP alone but far more effectively by either adenyl-XMP, the catalytic intermediate which is formed on exposure of the enzyme to ATP and XMP, or by the XMP–adenosine–PP_i combination which serves as an analog of the catalytic intermediate. This model more easily accounts for the effect of ammonia on the conformative response. The intermediate is subject to aminolysis and therefore its effectiveness would be expected to be reduced by ammonia to that of XMP alone. In contrast, it is unlikely that the XMP–adenosine–PP_i combination involves the formation of covalent bonds susceptible to aminolysis; thus, the effect of the combination would not be anatognized by ammonia.

Evidence of *qualitative* differences between the conformative response observed in the absence and in the presence of modifiers would favor the two-stage model. Although our present results show no indication of such differences, additional criteria for the characterization of the conformative response would have to be applied before the proposed models can be further evaluated.

The successful replacement of substrates by a noninteracting combination of ligands bears on the structural aspects of the conformative response. To take a specific case, our observations of the replacement of ATP by PP_i and adenosine indicate that the nucleoside is recognized only after the enzyme has bound both XMP and PP_i. This has been confirmed by direct binding studies. In view of the finding of a competitive relationship between adenosine and ATP, it is a reasonable assumption that this order of binding reflects the details of the alignment of the ATP molecule, terminal phosphate first, the nucleoside moiety next.

A further conclusion which follows from the replacement studies concerns the nature of the conformative response. Since the replacement assembly does not allow interaction among its elements, the different ligands, XMP, adenosine, PP_i, have to be assigned to distinct binding sites. Hence, the XMP-dependent binding of modifiers and the reciprocal effect of modifiers on the binding of XMP must be indirect and therefore mediated by the enzyme molecule, implying a conformational transition involving several distinct stereospecific sites.

We do not know to what extent this transition is necessary for the catalytic function of the enzyme. It would be interesting to study the effect of restriction of the conformative responsiveness of the enzyme on its catalytic properties. An indication of how this can be achieved comes from the observation that modified K_{cr} values based on rates of heat inactivation are considerably higher than expected on the basis of other determinations. We suggest that the conformational transition underlying modification of the conformative response is impaired at higher temperatures.

Another feasible approach to the relationship of the conformative response to the catalytic activity of XMP aminase involves analysis of mutant forms of the enzyme. While these enzymes have not suffered a loss of catalytic activity (Donovan *et al.*, 1967), preliminary results indicate that such mutations cause a significant reduction in the modification of the conformative response (N. Zyk, N. Citri, and H. S. Moyed, 1968, unpublished data).

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