depolymerization of the polymer. This possibility is currently being explored.

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Fructose Diphosphatase from Rabbit Liver. VII. Tyrosine Residues and Adenosine Monophosphate Inhibition*

S. Pontremoli, E. Grazi, and A. Accorsi

ABSTRACT: Treatment of crystalline rabbit liver fructose diphosphatase (FDPase) with *N*-acetylimidazole results in a time-dependent *O*-acetylation of ten tyrosyl residues.

In the first phase of the reaction two to three tyrosyl residues are acetylated with no change in catalytic properties. The acetylation of four additional residues is associated with loss of allosteric inhibition by adenosine monophosphate (AMP). Finally, with the acetylation of the last few residues the catalytic activity is abolished. The last phase is blocked by the substrate, fructose 1,6-diphosphate, while the inhibitor, AMP, protects the second group of tyrosyl residues. The evidence for acetylation of tyrosyl residues is based on spectral changes at 278 mμ and on the ability of hydroxylamine at pH 7.5 to reverse these effects. The characteristics of allosteric inhibition by AMP have been examined.

It is generally recognized that a critical and essentially irreversible step in gluconeogenesis is the conversion of fructose 1,6-diphosphate to fructose 6-phosphate, catalyzed by the enzyme FDPase.[1] Several lines of evidence point to a key role for this enzyme in the gluconeogenic sequence (Krebs *et al.*, 1964; Salas *et al.*, 1965; Fraenkel and Horecker, 1965). Evidence has also been obtained for the regulation of this enzyme, both *in vivo* (Weber *et al.*, 1965) and *in vitro* (Mangiarotti and Pontremoli, 1963; Pontremoli *et al.*, 1965a,b), by a number of biological compounds and chemical reagents.

An important regulatory mechanism (Taketa and Pogell, 1963; Newsholme, 1963) involves the specific and reversible allosteric inhibition of the enzyme by AMP. The authors have recently observed that treatment of crystalline rabbit liver FDPase with acetylimidazole leads to the acetylation of approximately ten tyrosyl residues with almost complete loss of catalytic activity which can be fully recovered by deacetylation with hydroxylamine at neutral pH (Pontremoli *et al.*, 1966).

However, kinetic analysis of the acetylation reaction has now shown that the partially acetylated enzyme, containing six *O*-acetyltyrosines, retains full catalytic activity but is almost completely desensitized against

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[1] Abbreviations used: FDPase, fructose diphosphatase; AMP, adenosine monophosphate; TPN^+, oxidized triphosphopyridine nucleotide; TPNH, reduced TPN.

TABLE I: Effect of AMP and Fructose 1,6-Diphosphate on the Acetylation of Fructose Diphosphatase by Acetylimidazole.[a]

	No Addition (A)			In the Presence of Fructose 1,6-Diphosphate (B)			In the Presence of AMP (C)		
Time (min)	*O*-Acetyltyrosine Formed (mole/mole)	Sp Act. (units/mg)	AMP Inhibn (%)	*O*-Acetyltyrosine Formed (mole/mole)	Sp Act. (units/mg)	AMP Inhibn (%)	*O*-Acetyltyrosine Formed (mole/mole)	Sp Act. (units/mg)	AMP Inhibn (%)
0	—	120	70	—	120	70	—	120	70
5	1.7	120	69	0.7	120	70	0.72	120	68
10	3.0	120	54	1.5	120	67	1.5	120	70
20	6.2	113	15	3.6	120	48	2.1	120	67
40	7.8	67	—	4.4	115	30	3.0	96	68
60	10.2	6	—	6.0	113	16	4.8	54	70
80	10.1	6	—	6.15	111	16	6.4	18	66

[a] FDPase (3 mg/ml, sp act. 120 units/mg of protein) was dissolved in 0.05 M borate buffer, pH 7.5. Acetylimidazole (0.75 mg/ml) was added at the beginning of the experiment and after 20, 30, and 50 min. In expt B the acetylation was conducted in the presence of 1 mM fructose diphosphate; in that of column C in the presence of 1.0 mM AMP. At the times indicated, samples were taken and dialyzed to remove the reaction products. The catalytic activity was assayed at pH 9.1 with Mn^{2+}; the inhibition by 0.5 mM AMP was followed at pH 7.5 with Mn^{2+} in the conditions indicated under methods. The number of tyrosines acetylated was determined by the decrease in the absorbance at 278 mμ as described under Methods.

allosteric inhibition by AMP. In all other respects the properties of the enzyme remain unchanged. It yields the same value for the K_m for fructose 1,6-diphosphate, shows the same pH–activity curve, and is unchanged with respect to molecular size. It is still sensitive to inhibition by excess fructose 1,6-diphosphate. When four additional tyrosyl residues are acetylated, the catalytic activity is lost. Fructose 1,6-diphosphate protects against acetylation of these four tyrosyl residues; in its presence only the allosteric properties are affected by exposure to acetylimidazole.

While this manuscript was in preparation a similar effect on allosteric properties and catalytic activity was reported by Rosen and Rosen (1966). These workers employed fluorodinitrobenzene to characterize tyrosyl and lysyl residues at the catalytic and allosteric site of FDPase isolated from *Candida utilis*.

Experimental Section

The procedure for the preparation of the crystalline enzyme and the reagents used has been described in previous papers of this series (Pontremoli *et al.*, 1965c, 1966). Fructose diphosphatase activity was measured spectrophotometrically by following the rate of TPNH formation at 340 mμ in the presence of excess phosphoglucose isomerase and glucose 6-phosphate dehydrogenase. The usual test system (1 ml) contained 0.1 mM fructose 1,6-diphosphate, 1 mM $MnCl_2$ (or 10 mM $MgCl_2$), 40 mM triethanolamine buffer, pH 7.5, containing 0.5 mM EDTA (or 40 mM glycine buffer, pH 9.1, without EDTA), 0.15 mM TPN^+, 2 units of glucose 6-phosphate isomerase, and 0.3 unit of glucose 6-phosphate dehydrogenase. The temperature was 22°. Unless otherwise indicated the enzymatic analyses have been performed with $MnCl_2$ in glycine buffer at pH 9.1. One unit of enzyme was defined as the amount which would cause an optical density change of 1.0/min under the above conditions. Specific activity is expressed as units per milligram of protein.

Protein concentration was measured with Folin reagent (Lowry *et al.*, 1951) standardized against a known dry weight of dialyzed crystalline FDPase. The reaction with acetylimidazole was carried out at 22° in 0.05 M borate buffer, pH 7.5, with a protein concentration of 3 mg/ml. The reagent was usually added in four portions (0.75 mg/ml each) at the beginning of the incubation and after 20, 30, and 50 min. The total amount of acetylimidazole added corresponded to a 1200-fold molar excess over the molar quantity of protein enzyme. The acetylated protein was precipitated with ammonium sulfate (70% saturation), dissolved in water, and dialyzed against distilled water at 2° for 90 min before analysis. Control samples were treated in the same manner but without addition of acetylimidazole. Acetylation of tyrosine residues was estimated with a Zeiss Model PMQII spectrophotometer on the basis of a decrease in molar absorbance at 278 mμ of 1160 between *N*-acetyltyrosine and *N,O*-diacetyltyrosine (Simpson *et al.*, 1963). The determinations were made in 10 mM Tris buffer, pH 7.5, containing 1 M NaCl; the protein concentration was usually 0.3 mg/ml.

The alkaline and neutral hydroxamate procedures

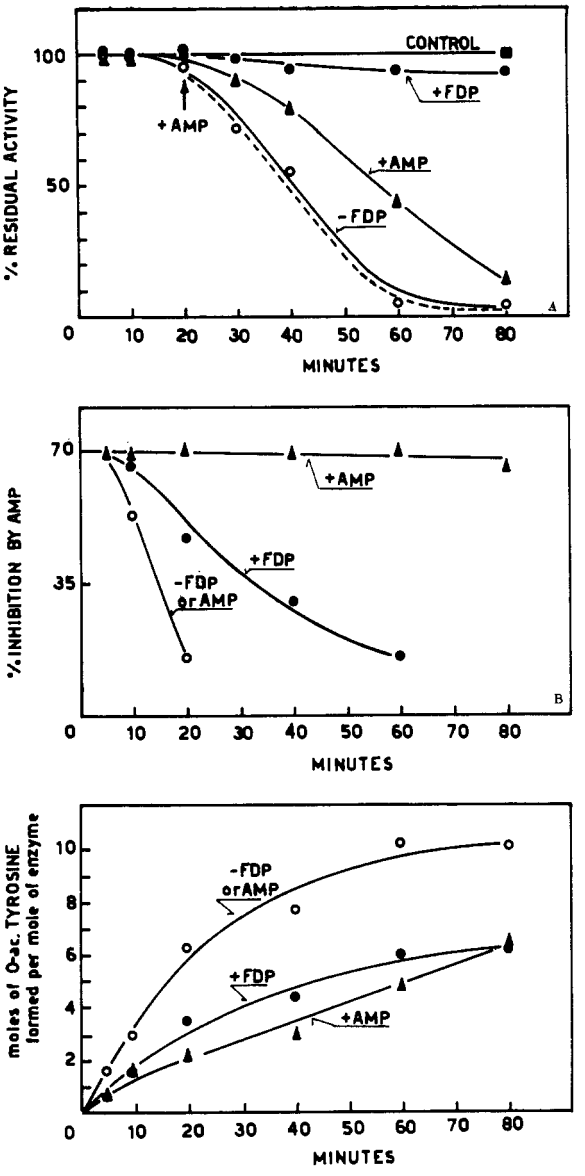

FIGURE 1: Effect of AMP and fructose 1,6-diphosphate on the acetylation of FDPase by acetylimidazole. The data in the figure are taken from Table I. (A) FDPase activity. (B) Inhibition by 0.5 mM AMP. (C) *O*-Acetylation of tyrosyl residues per mole of enzyme. Control sample (■); sample treated with acetylimidazole alone (○); sample treated with acetylimidazole in the presence of 1 mM fructose diphosphate (●) or 1 mM AMP (▲).

of Hestrin (1949) as modified by Balls and Wood (1956) were employed to measure *O*-acetylation. Sulfhydryl groups were measured spectrophotometrically by titration with *p*-hydroxymercuribenzoate according to Boyer (1954). The degree of modification of the free amino groups was estimated by means of the ninhydrin reaction (Moore and Stein, 1954).

Results

Changes in Catalytic and Allosteric Properties on Acetylation. Treatment of the enzyme with acetylimidazole results in the progressive acetylation of tyrosyl residues up to an approximate total number of ten. As will be shown later, acetylation of amino, imidazole, aliphatic hydroxyl, or sulfhydryl residues has been excluded or was found not to be correlated with the observed changes in catalytic properties. The time- and concentration-dependent acetylation of the enzyme appeared to occur in three phases (Table IA). There was an initial acetylation of approximately two to three tyrosyl residues which left unchanged both the catalytic activity and the sensitivity to inhibition by AMP, followed by the subsequent acetylation of three to four additional tyrosyl residues which resulted in almost complete desensitization to AMP inhibition with little or no loss in hydrolytic activity. Finally the acetylation of four more tyrosyl residues was accompanied by loss of enzymatic activity.

If acetylation was carried out in the presence of 1 mM fructose 1,6-diphosphate (Table IB) protection against acetylation and loss of catalytic activity was observed. Under these conditions only six tyrosyl residues were acetylated and the enzyme retained full catalytic activity.

Fructose diphosphatase was, however, largely desensitized against AMP inhibition. Analysis of the relationship between extent of acetylation and AMP desensitization shows a similarity in the results obtained in the presence or absence of fructose 1,6-diphosphate (Table IA and B). When less than two tyrosyl residues were *O*-acetylated no changes were observed in the allosteric properties, while the acetylation of four additional residues was accompanied by almost complete desensitization, with little loss of catalytic activity.

When acetylation was carried out in the presence of AMP (Table IC) protection against acetylation was somewhat more effective than in the presence of fructose 1,6-diphosphate.[2] In this case the total number of tyrosyl residues acetylated was again six, but at this stage the enzyme had almost completely lost its catalytic activity, still retaining the property of being inhibited by AMP.

Kinetic analysis of the result of exposure to acetylimidazole (Figure 1) reveals that fructose 1,6-diphosphate is more efficient than AMP in protecting against the loss of catalytic activity (Figure 1A), while the latter is much more efficient in protecting against the loss of allosteric inhibition (Figure 1B). The kinetics of acetylation of tyrosyl residues is similar whether the substrate or the inhibitor is present during exposure to the acetylating agent (Figure 1C). It is of interest

[2] The protection by fructose diphosphate and AMP is not accounted for by reagent subtraction. In fact, in a control system, the presence of either AMP or fructose diphosphate does not affect the rate of reaction of acetylimidazole with the amino acid tyrosine as judged by the decrease in optical density at 280 mμ.

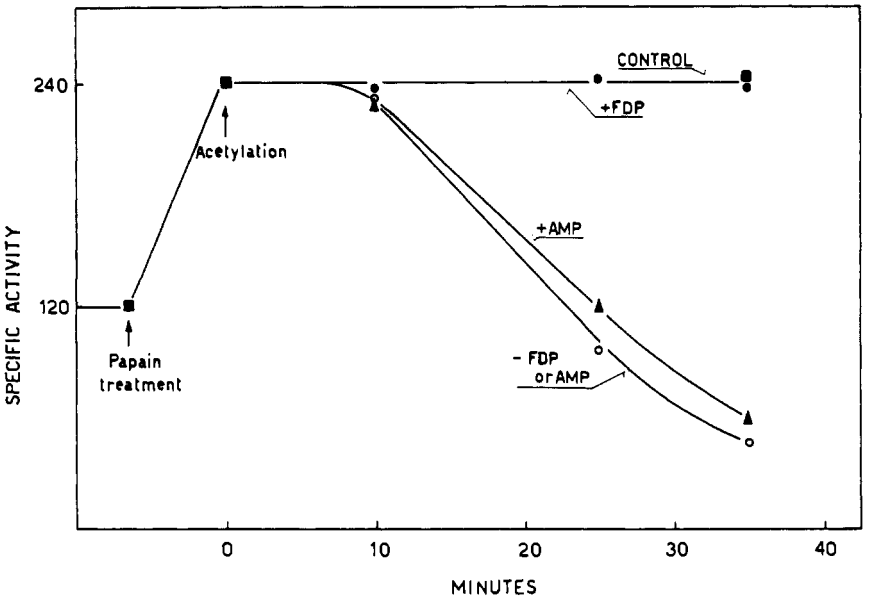

FIGURE 2: Acetylation of papain-treated FDPase. Treatment with papain was performed at pH 5.5 and 22°. FDPase, 12 mg (sp act. 120 units/mg of protein), was dissolved in 3 ml of 0.02 M acetate buffer, pH 5.5, and treated with 0.6 mg of papain. After 90 min the protein was precipitated with 70% saturated ammonium sulfate and dissolved in 1 ml of water, and papain was removed by gel filtration through a Sephadex G-100 column (2.5 × 52 cm) equilibrated with 0.05 M Tris buffer, pH 8.7, plus 0.1 M KCl. The fractions containing FDPase (free of papain) were pooled and the protein was precipitated with 70% saturated ammonium sulfate, dissolved in 1 ml of water, and dialyzed against water. Papain-treated FDPase was divided into four aliquots and treated with acetylimidazole alone, with acetylimidazole plus 1 mM fructose 1,6-diphosphate, and with acetylimidazole plus 1 mM AMP. The fourth aliquot was utilized as a control sample and received no treatment. The reaction with acetylimidazole (1 mg/mg of protein) was carried out for 35 min in 0.05 M borate buffer, pH 7.5, and with an enzyme concentration of 3 mg/ml. The inhibition by AMP on the original native enzyme was 70% and was reduced to 14% after treatment with papain. At the end of the treatment with acetylimidazole (35 min) the number of tyrosyl residues acetylated per mole of enzyme was estimated and found to be equal to 4.7 in the sample acetylated in the presence of fructose 1,6-diphosphate, to 9.8 in the sample acetylated in the presence of AMP, and to 9.7 in the sample acetylated in the absence of either fructose 1,6-diphosphate or AMP.

that equal total numbers of tyrosyl residues are acetylated in the presence of fructose 1,6-diphosphate or AMP although the enzymatic properties of the products formed under these conditions are significantly different. The result suggests that different groups (or portions of the protein) are responsible for inhibition by AMP and for catalytic activity.

Approximately four tyrosyl residues seem to be involved in each specific function. These tyrosyl groups may be directly responsible for the binding of fructose 1,6-diphosphate and AMP or, alternatively, they may be essential for maintenance of the required conformation of the catalytic and allosteric sites. In the presence of fructose 1,6-diphosphate, acetylation is slower and more time is required to acetylate the groups responsible for allosteric sensitivity. Conversely, AMP affords some protection against inactivation, indicating the presence in FDPase of distinct but reciprocally interacting regions for binding of AMP and of fructose 1,6-diphosphate. The protective effect of AMP against inactivation of the enzyme appears to be lost in coincidence with the desensitization to allosteric inhibition.

Acetylation of FDPase after Exposure to Papain. In order to further investigate the question of a mutual relationship between catalytic and allosteric sites, the authors have studied the effect of acetylimidazole on FDPase treated with papain. Papain treatment has been shown to produce an increase in FDPase activity measured at pH 9.1 and a decrease at pH 7.5. These changes in catalytic activity are accompanied by a loss of sensitivity to AMP inhibition (Taketa and Pogell, 1965).

As shown in Figure 2 the specific activity of a crystalline preparation of rabbit liver FDPase was increased approximately twofold after papain treatment. Papain-treated FDPase lost 90% of its catalytic activity on acetylation with acetylimidazole. Fructose 1,6-diphosphate fully protected against this inactivation whereas AMP failed to show the protective effect (Figure 2) which, as previously shown (Table IC), is always present in the native enzyme. This result confirms the allosteric

TABLE II: Acetylation of FDPase and Deacetylation with Hydroxylamine.[a]

	Sp Act.	AMP Inhibn (%)	*O*-Acetyl-tyrosine (mole/mole)	Hydroxamate (mole/mole)		Free Thiol Groups (mole/mole)	Acetylated Amino Groups (%)
				pH 7.5	pH 11.5		
Native FDPase	120	70	—	—	—	20	—
Acetylated 5 min, unprotected (A)	120	70	1.7	—	—	20	—
Acetylated 60 min, unprotected (B)	8	10	10.2	—	—	20	9
Acetylated 60 min, protected AMP (C)	125	16	6.0	—	—	20	8
Acetylated 80 min, protected by FDP (D)	18	68	6.4	—	—	20	9
A + hydroxylamine	125	70	—	1.8	1.6	20	—
B + hydroxylamine	120	68	—	10.4	10.5	20	9
C + hydroxylamine	110	67	—	5.8	6.0	20	9
D + hydroxylamine	120	72	—	6.5	6.4	20	8

[a] The samples analyzed in this table are those described in Table I. FDPase was incubated with acetylimidazole for (A) 5 min and (B) for 60 min; (C) FDPase incubated with acetylimidazole for 60 min in the presence of 1 mM AMP; (D) FDPase incubated with acetylimidazole for 80 min in the presence of fructose 1,6-diphosphate (FDP), 1 mM. Analysis of catalytic activity was performed at pH 9.1, inhibition by 0.5 mM AMP was measured at pH 7.5, both with Mn^{2+} as described under Methods. Deacetylation with hydroxylamine was performed at pH 7.5 and 22° for 20 min in a reaction mixture containing 1 M NH_2OH, 1 M NaCl, and 0.01 M Tris buffer. Catalytic activity and inhibition by AMP were determined directly after dilution of aliquots from the deacetylation reaction mixture. The final concentration of hydroxylamine in the assay mixture was 2 mM. All the other analytical procedures were performed as described under Methods.

mechanism of AMP inhibition and the reciprocal structural relationship between the substrate and allosteric binding sites in FDPase.

Reversal of Acetylation Effects. Treatment of acetylated FDPase with hydroxylamine at neutral pH results in deacetylation of the *O*-acetyl residues accompanied by the recovery of all of the enzymatic properties of the native enzyme (Table II). Deacetylation was followed by the spectral changes at 278 mμ, and excellent correlation between acetohydroxamate formation at pH 7.5 and tyrosine acetylation as calculated from the spectral changes was observed. There was no evidence for acetylation of aliphatic hydroxyl or sulfhydryl groups. A few amino groups were acetylated under our experimental conditions, but these did not seem to participate in the reversible changes in enzyme properties. The difference spectra in the region 240–250 mμ for the various samples of acetylated FDPase compared with native FDPase did not indicate the formation of acetylhistidyl residues.

Inhibition of Rabbit Liver FDPase by AMP. In connection with these studies, a detailed analysis of the effect of AMP concentration at neutral and alkaline pH was undertaken (Figure 3). The same degree of inhibition was observed if, prior to the measurement of catalytic activity, the enzyme was preincubated with AMP for 10 min, or if AMP was added directly to the complete incubation mixture prior to the addition of fructose 1,6-diphosphate. Inhibition was slightly more pronounced in the presence of Mg^{2+} than in the presence of Mn^{2+}. In the untreated sample, 0.1 mM AMP caused 43% inhibition in the presence of Mn^{2+}, and 64% in the presence of Mg^{2+}. Under these conditions the enzyme samples acetylated for 20 min without or 60 min in the presence of the substrate, and both containing six acetylated tyrosyl residues, were almost unaffected by AMP.

Inhibition by AMP at pH 9.1 was much less than at pH 7.5, especially with Mn^{2+} as the activating cation (Figure 2). Similar differences in sensitivity to AMP at neutral and alkaline pH have been reported by Taketa and Pogell (1965) for the partially purified rabbit liver preparation. However, desensitization against AMP inhibition by acetylation and protection against this loss of allosteric inhibition by AMP were also present when the enzyme was tested at pH 9.1.

Discussion

It is evident from these results and those previously reported (Pontremoli *et al.*, 1966) that acetylation of tyrosyl residues results in significant changes in the enzymatic properties of crystalline rabbit liver FDPase. Since the acetylation process is concentration and time

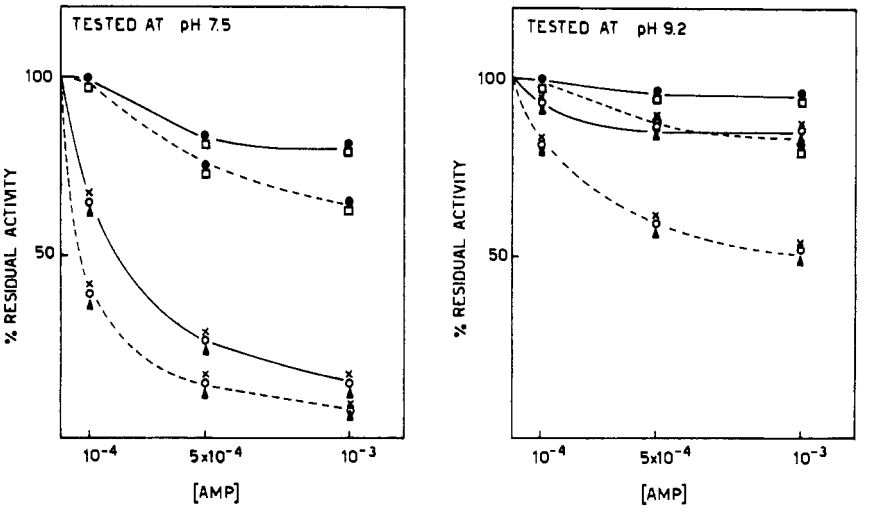

FIGURE 3: Inhibition of acetylated FDPase by AMP at pH 7.5 or 9.1. The samples analyzed were prepared as described in the experiment described in Table I. Native enzyme (O); FDPase treated with acetylimidazole for 5 min (▲) and for 20 min (□); FDPase treated with acetylimidazole in the presence of 1 mM fructose 1,6-diphosphate for 60 min (●) or in the presence of 1 mM AMP for 80 min (×). Assayed in the presence of 10 mM Mg^{2+} (- - - -) and of 1 mM mM^{2+} (——) as described under Methods.

dependent, it has been possible to correlate these changes to the nature and number of functional groups acetylated. Since the changes in catalytic and allosteric properties are correlated with a decrease in absorbance at 278 mμ and since hydroxylamine at neutral pH restored the original properties of the enzyme, it is apparent that phenolic hydroxyl groups of tyrosine are involved.

The quantitative data indicate that reversible desensitization of the allosteric inhibition by AMP is associated with acetylation of four tyrosyl residues and, since AMP protects against this desensitization effect, it is reasonable to assume that it prevents the acetylation of these tyrosyl residues. This may in some way be related to the observation of Taketa and Pogell (1965) who found that 3–4 moles of AMP is bound/mole of enzyme.

It is clear that in FDPase isolated from rabbit liver the site(s) responsible for inhibition by AMP is distinct from these involved in catalytic activity. The existence of two forms of acetylated FDPase (both containing six *O*-acetylated tyrosines)—one retaining full catalytic activity but desensitized against AMP inhibition, and the other almost completely inactivated but retaining full allosteric inhibition—is a direct demonstration for the existence of these two distinct regions. A similar conclusion was reached by Rosen and Rosen (1966) with the enzyme from *C. utilis*.

The reciprocal partial protection by AMP and fructose 1,6-diphosphate against inactivation and desensitization, respectively, must therefore be a consequence of interaction of these sites. The finding that papain-treated enzyme, in which the inhibition by AMP is almost completely lost with a change in the apparent *n* value for AMP to 1 (Taketa and Pogell, 1965), is no longer protected against loss of enzymatic activity by AMP on acetylation confirms the allosteric nature of the inhibition by adenylic acid. This is also indicated by the fact that desensitization of the enzyme by acetylation is accompanied by a loss of the protective effect of AMP against inactivation.

The inhibitory effects of AMP on the crystalline enzyme are similar to those reported by Taketa and Pogell (1965) with partially purified preparations. Maximum inhibition is obtained at neutral pH although desensitization is observed when the enzyme is tested throughout the pH range.

Further work is in progress to investigate the structural relationship of the two regions in FDPase which are involved in catalytic and allosteric activity. It is of particular interest to determine whether the groups affected are on different chains since we have previously reported evidences for two types of subunits which can be dissociated at acid pH (Pontremoli *et al.*, 1966).

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The Reaction of Diazonium-1H-tetrazole with Proteins. Determination of Tyrosine and Histidine Content*

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ABSTRACT: Coupling of diazonium-1H-tetrazole (DHT) with histidine and tyrosine and quantitative conversion to the respective bisazo derivatives serve as the basis of a method for the determination of these residues in proteins. This reagent, previously proposed for the determination of reactive histidyl residues (Horinishi, H., Hachimori, Y., Hurihara, K., and Shibata, K. (1964), *Biochim. Biophys. Acta 86*, 477), forms monoazo- and bisazotyrosine and -histidine derivatives each of which exhibits characteristic spectra. These differ to some extent from those of the respective *N*-acylamino acids and peptides. Their wavelengths of maximal absorption and molar absorptivities have been characterized and assigned and serve as the basis for the quantitative method proposed. The spectral characteristics of the peptides or acylamino acids both of tyrosine and of histidine have been found to be the proper standards of reference for the determination of the respective residues in proteins.

The reactivity of the proteins and these spectral characteristics of the derivatives combine to render results obtained on native proteins ambiguous: three of the possible azo derivatives, *i.e.*, mono- and bisazotyrosine and bisazohistidine, all absorb at 480 mμ, the wavelength previously employed for the measurement of histidine alone. Complete conversion at high DHT concentration of both monoazo to the respective bisazo species, combined with alkali denaturation of proteins prior to coupling, leads to a quantitative method for the simultaneous determination of the tyrosine and histidine contents of proteins by photometry at two wavelengths.

Diazonium compounds have been employed extensively to modify proteins, to study composition and structure (Howard and Wild, 1957; Higgins and Harrington, 1959; Tabachnick and Sobotka, 1960) and their relationship to function of enzymes (Fraenkel-Conrat *et al.*, 1949; Gundlach *et al.*, 1962), and also to produce specific antigenic determinants (Landsteiner, 1945). Diazonium compounds couple readily with histidyl, tyrosyl, and lysyl residues of proteins, but both the lack of specificity and the incomplete resolution of the spectral bands which accompany the formation of different azo derivatives of amino acids have restricted interest in their use as site-specific reagents.

Recently, Horinishi *et al.* (1964) have examined diazonium-1H-tetrazole[1] and proposed its use as a coupling reagent for proteins. The absorption bands of monoazotyrosine, bisazohistidine, and bisazotyro-

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[1] Abbreviation used: DHT, diazonium-1H-tetrazole.