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Effect of Adenosine Monophosphate on the Reactivity of Thiol Groups of D-Fructose 1,6-Diphosphatase from Rabbit Liver*

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ABSTRACT: Adenosine monophosphate, an allosteric inhibitor of most fructose 1,6-diphosphatases, markedly reduces the reactivity of SH groups of rabbit liver fructose 1,6-diphosphatases toward 5,5′-dithiobis(2-nitrobenzoic acid) and 2,2′-dithiodipyridine at pH 7.5. Six out of the twenty SH groups react with 5,5′-dithiobis(2-nitrobenzoic acid) in the absence of added ligand, but only two groups react when 0.5 mM adenosine monophsophate is present. This effect is highly specific for adenosine monophosphate and deoxyadenosine monophosphate and is reversed by ethylenediaminetetraacetic acid, an activator of the enzyme. The activation by 2,2′-dithiobisethylamine and 2-hydroxyethyl disulfide is also diminished by adenosine monophosphate. The addition of the substrate D-fructose 1,6-diphosphate does not affect the reactivity of SH groups by itself, but it does exert a synergistic effect with adenosine monophosphate.

The presence of substrate, however, is not obligatory for the demonstration of altered SH reactivity in the presence of adenosine monophosphate. The disulfide reagents provide a sensitive indication that the enzyme may exist in an altered conformational state in the presence of its allosteric inhibitor.

Mammalian liver FDPase[1] catalyzes the conversion of fructose 1,6-diphosphate into fructose 6-phosphate in the presence of Mg^{2+} or Mn^{2+} and plays an essential role in the process of gluconeogenesis. Its catalytic activity is inhibited by AMP. This inhibition is reversible, noncompetitive with substrate, and maximal at neutral pH (Mendicino and Vasarhely, 1963; Gancedo *et al.*, 1965; Taketa and Pogell, 1965). Chemical modification of FDPases purified from rabbit liver and *Candida utilis* have indicated that the inhibition due to AMP occurs at sites on the enzyme which are distinct from those involved in catalysis (Horecker *et al.*, 1966; Pontremoli *et al.*, 1966; Rosen and Rosen, 1966). Furthermore, the FDPase isolated from *C. utilis* is dissociated into subunits by the addition of AMP at pH 9.5 (Rosen *et al.*, 1967). This drastic alteration in the quaternary structure at pH 9.5 may represent an exaggerated manifestation of subtle conformational changes exhibited by the enzyme in the presence of AMP at neutral pH. The FDPase isolated from rabbit liver, however, does not dissociate under these

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[1] Abbreviations used are: PMB, *p*-mercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FDPase, fructose 1,6-diphosphatase.

conditions. In an effort to demonstrate conformational changes occurring in the presence of AMP at neutral pH, we have examined the reactivity of the SH groups of rabbit liver FDPase in the presence and absence of the ligand. The results suggest that in the presence of the allosteric inhibitor the enzyme may exist in an altered conformational state.

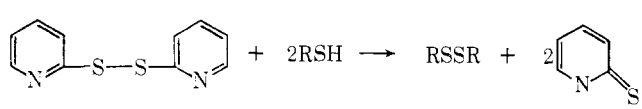
Materials and Methods

Frozen rabbit livers were obtained from Pel-Freeze Biologicals, Rogers, Ark. DTNB,[1] iodoacetamide, and ^{14}C-labeled PMB with the radioactive label in the carboxyl group were products of Calbiochem. 2-Mercaptopyridine and 2-hydroxyethyl disulfide were from Aldrich Chemical Co.; 2,2′-dithiobisethylamine dihydrochloride was obtained from Mann Biochemicals; PMB and bacterial alkaline phosphatase were products of Sigma Biochemicals. Other chemicals were of the best commercial grade available. FDPase was prepared from rabbit liver using the procedure of Pontremoli *et al.* (1965b). The purified enzyme catalyzed the conversion of 22.5 μmoles of FDP into F-6-P/min per mg of protein at pH 9.5. The enzyme was assayed spectrophotometrically by following the formation of reduced NADP at 340 mμ in the presence of FDP, Mn^{2+}, and excess hexose phosphate isomerase and glucose 6-phosphate dehydrogenase. At pH 7.5, the enzyme was assayed in 0.1 M triethanolamine–HCl buffer (Pontremoli *et al.*, 1965b). Protein was determined by absorbancy at 280 mμ or according to Lowry *et al.* (1951). Radioactive PMB was diluted with carrier PMB and purified by repeatedly precipitating with 1 N HCl and dissolving the pellet in 1 N NaOH. After two cycles, the precipitate was dissolved in 0.2 M glycylglycine buffer (pH 7.8), and its concentration was determined spectrophotometrically (Boyer, 1954). Radioactivity was determined using a Nuclear-Chicago gas-flow counter.

2,2′-Dithiodipyridine was prepared by iodination of 2-mercaptopyridine (Marckwald *et al.*, 1900) followed by two recrystallizations from petroleum ether (bp 30–60°). The crystalline product (mp 57°) was stored dry in the cold.

Titration of SH Groups. TITRATION WITH DTNB. The procedure employed was similar to that of Ellman (1959), except that 0.1 M triethanolamine–HCl buffer (pH 7.5), was used instead of phosphate buffer (pH 8.0). In this buffer, the thionitrobenzoate liberated by the reaction of DTNB with SH groups had an ε of 12,500 at 412 mμ (using cysteine–HCl as standard) in contrast to the value of 13,600 in phosphate buffer at pH 8.0 as reported by Ellman.

TITRATION WITH 2,2′-DITHIODIPYRIDINE. Ther eagent has been employed by Grasetti and Murray (1967) to titrate SH groups in Ringer–phosphate solutions (pH 7.0–8.0). The reaction proceeds as follows



R = protein

The thiopyridone liberated can be estimated at 343 mμ (ε 7.06 × 10^3). In contrast to DTNB, which reacts by a disulfide-exchange mechanism, 2,2′-dithiodipyridine reacts by formation of an inter- or intramolecular disulfide. In the experiments to be reported here, the reagent was dissolved in dimethylformamide. Upon addition of the reagent to the enzyme, there was a relatively slow increase in absorbancy at 343 mμ followed by a rapid rise associated with the appearance of turbidity. The latter may be due to aggregation brought about by intra- or intermolecular disulfide formation. The number of SH groups titrated was calculated from the absorbancy of the thiopyridone at 343 mμ after removal of the aggregated protein by passage through a small column of DEAE-Sephadex.

Reaction with ^{14}C-Labeled PMB. An aliquot of ^{14}C-labeled PMB, equivalent to a twofold molar excess of the number of SH groups in the enzyme, was added to the sample at 24° in 0.1 M Tris–HCl buffer (pH 7.5). After 2 hr, the reaction mixture was passed through a column of Sephadex G-50 (0.9 × 40 cm) equilibrated with the Tris–HCl buffer. Fractions (1 ml) were collected at the rate of 30 ml/hr. The protein content and the radioactivity of each fraction were then determined.

Reaction with Iodoacetamide. Iodoacetamide in triethanolamine–HCl buffer (pH 7.5) was added in 100-fold excess with respect to the enzyme. After a specified period at 24°, the reaction mixture was dialyzed against water for 48 hr, lyophilized, and hydrolyzed in constant-boiling HCl (5.7 N) at 110° for 22 hr. *S*-Carboxymethylcysteine was determined by amino acid analysis (Spackman *et al.*, 1958) in a Spinco 120B analyzer using the alanine and aspartic acid content of the enzyme (Pontremoli *et al.*, 1965a) as internal standards.

Activation Experiments. Activation of the enzyme in the presence of 2,2′-dithiobisethylamine dihydrochloride and 2-hydroxyethyl disulfide was carried out according to Pontremoli *et al.* (1967). The activity of the enzyme was measured in the absence of EDTA, at pH 7.5 in triethanolamine–HCl buffer.

Results

In the absence of AMP, a relatively rapid reaction of FDPase with DTNB corresponding to the titration of approximately four SH groups is observed. This is followed by a slow reaction corresponding to the titration of two more SH groups.

In the presence of 0.5 mM AMP the first SH group is titrated in a manner similar to the reaction observed in the absence of AMP, followed by a slow reaction representing the titration of only one additional group. The possibility that the decreased reactivity in the presence of AMP is due to heavy metal impurities in the sample of AMP is excluded by the following results: (a) the reaction of DTNB with cysteine is not affected by the presence of AMP; (b) AMP which had been passed through a column of chelating resin (Chelex) produced the same effect; and (c) addition of sodium dodecyl sulfate (final concentration 0.3 mM) to the reaction mixture in the presence as well as in the absence of AMP increased the number of SH groups titrated to 20, which approximates the total number of SH groups in the enzyme (Pontremoli *et al.*, 1965a).

The Effect of Other Nucleotides and the Substrate on the Reaction of SH Groups with DTNB. The observed effect of AMP on the reactivity of SH groups was highly specific (Table I). In the presence of FDP, approximately four groups are titrated. Of the nucleotides tested, only dAMP, which has an inhibitory effect on the enzyme identical with that of

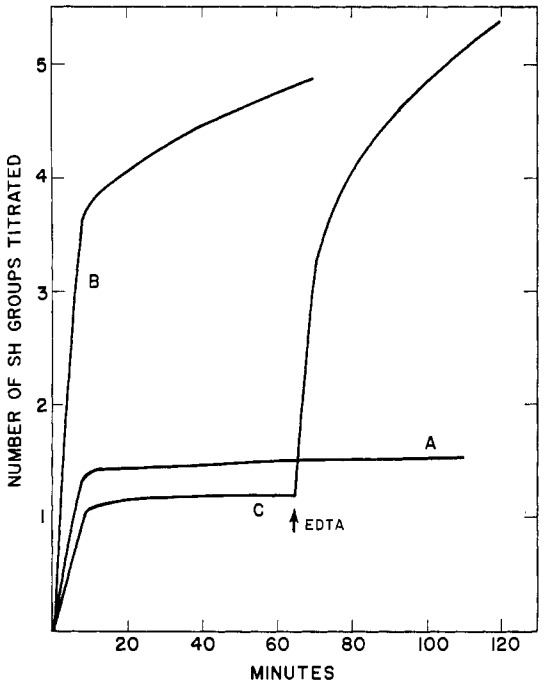


FIGURE 1: Reversal by EDTA of the effect of AMP on the titratability of SH groups. The conditions of the reaction are described in the footnote to Table I. (A) Titration in the presence of 0.5 mM AMP. (B) Titration in the presence of 0.5 mM AMP and 0.5 mM EDTA. (C) Titration in the presence of 0.5 mM AMP, with EDTA added at the time indicated by the arrow.

TABLE I: Effect of Ligands on the Titration of SH Groups by DTNB.[a]

Ligand	Concn (mM)	No. of SH Groups Titrated/Mole of FDPase in 120 Min
None		6.35
None + sodium dodecyl sulfate (3 mM)		20.50
AMP	0.50	1.90
AMP + sodium dodecyl sulfate (3 mM)	0.50	20.50
dAMP	0.50	1.94
ADP	0.50	5.95
GMP	0.50	6.34
IMP	0.50	6.25
UMP	0.50	5.93
CMP	0.50	7.12[b]
FDP	0.10	4.51
FDP	0.50	4.16
FDP	1.00	3.80
FDP (Chelex treated)	0.10	3.80

[a] The reaction mixture (1.0 ml) contained 100 μmoles of triethanolamine–HCl buffer (pH 7.5) and 125 mμmoles of DTNB. The reaction was initiated by the addition of enzyme (3 mμmoles). Absorbancy at 412 mμ was recorded automatically in a Gilford 2000 recorder coupled to a Beckman spectrophotometer. [b] On four subsequent determinations values averaging 6.4 SH groups were obtained.

AMP, affected the reactivity of SH groups. The divalent cations, Mg^{2+} and Mn^{2+}, did not markedly affect the reactivity of the SH groups.

Effect of EDTA. EDTA reversed the effect of AMP on the reactivity of SH groups. In the presence of 0.5 mM AMP, four groups reacted readily when EDTA was added at the beginning of the reaction. At this concentration of AMP, with no EDTA added, one to two groups were titrated. After one group had been titrated in the presence of 0.5 mM AMP, the addition of EDTA caused a rapid reaction of SH groups (Figure 1). EDTA had no effect on the reactivity of SH groups titrated in the absence of AMP. In addition, it did not affect the color reaction of DTNB with cysteine.

Studies with Iodoacetamide and [^{14}C]PMB. The influence of AMP on the reactivity of SH groups toward these reagents is shown in Table II. In the presence of AMP, there is a slight but distinct decrease in the reactivity of these groups toward iodoacetamide. With [^{14}C]PMB the number of groups titrated in the presence or absence of AMP is close to the value of 20 reported by Pontremoli *et al.* (1965a) who employed the direct spectrophotometric method of Boyer (1954).

Reduction of FDPase with 2,2′-Dithiodipyridine. The course of reaction of the SH groups of FDPase with 2,2′-dithiodipyridine is illustrated in Figure 2. The absorbancy at 343 mμ did not reach a plateau, but continued to rise. When the aggregated protein was removed after 60 min of reaction, the absorbancy of the thiopyridone formed corresponded to a titration of 17–18 SH groups. There was no apparent protein aggregation when the titration was performed in the absence of 0.5 mM AMP. Removal of protein and determination of the thiopyridone demonstrated the titration of five SH groups. Addition of EDTA (final concentration 0.5 mM) resulted in a rapid rise in absorbancy followed by aggregation. Under these circumstances, about 15 groups were titrated.

Activation of FDPase by Dithiobisethylamine and Its Inhibition by AMP. Pontremoli *et al.* (1967) observed that the reaction of FDPase with a variety of disulfides resulted in a threefold activation of the enzyme without loss of sensitivity to inhibition by AMP. Since the reduction in the reactivity of SH groups in the presence of AMP is most pronounced with the disulfides DTNB and 2,2′-dithiodipyridine, it was of interest to determine whether the reactions of other disulfides with the SH groups of the enzyme are affected by the presence of AMP. Dithiobisethylamine was chosen for this experiment since this compound was studied in detail by Pontremoli *et al.* (1967). Activation of the enzyme at pH 7.5 was used as the criterion for reactivity of SH groups. The activation pattern observed in the absence of AMP is similar to that obtained by Pontremoli *et al.* (1967). The addition of AMP (1.0 mM) diminished the activation by approximately 50% (Figure 3).

Synergistic Effect of FDP and AMP on the Reaction of FDPase with DTNB. Watanabe *et al.* (1968) have shown that the binding of AMP to FDPase is enhanced by the substrate, FDP. It was of interest, therefore, to find out whether the addition of FDP would enhance the inhibitory effect of

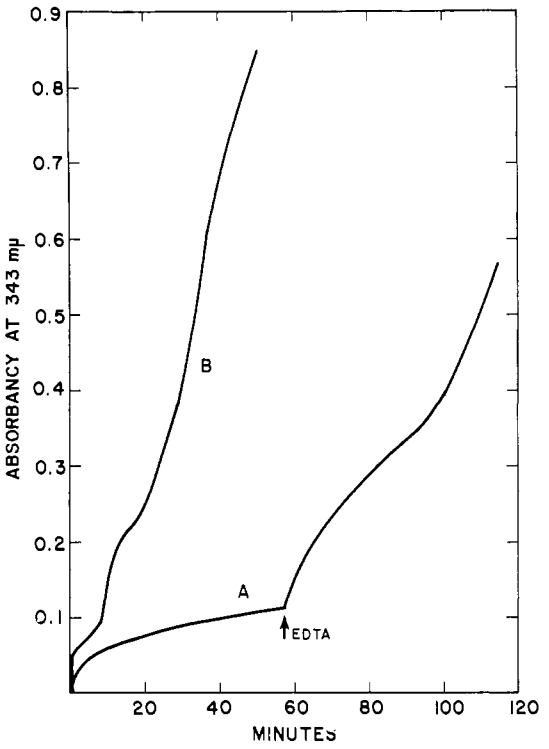


FIGURE 2: Aggregation of FDPase in the presence of 2,2′-dithiodipyridine. 2,2′-Dithiodipyridine (1 μmole) in 10 μl of dimethylformamide was added to 1 ml of 0.1 M triethanolamine–HCl buffer containing 10% (v/v) dimethylformamide and 0.5 μmole of AMP. The reaction was initiated by the addition of 3 mμmoles of enzyme. AMP was omitted in control experiments. (A) Reaction carried out in the presence of AMP with the addition of EDTA (0.5 μmole) at time indicated by the arrow. (B) Reaction carried out in the absence of AMP.

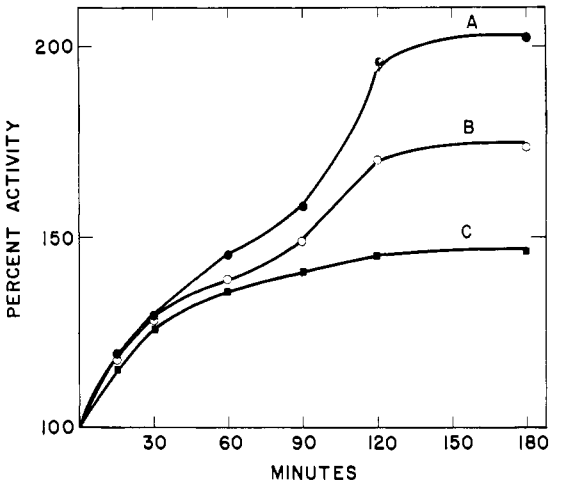


FIGURE 3: Effect of AMP on the activation of FDPase by dithiobisethylamine dihydrochloride. The reaction mixture (1.0 ml) contained 3 mμmoles of enzyme, AMP (0.1 mM or 1.0 mM), and 0.3 μmole of dithiobisethylamine dihydrochloride. The enzyme was assayed at pH 7.5 as described in the text. (A) Reaction carried out in the absence of AMP. (B) Reaction carried out in the presence of 0.1 mM AMP. (C) Reaction carried out in the presence of 1.0 mM AMP.

AMP on the reaction of the enzyme with DTNB. The results are shown in Figure 4. In the presence of relatively low concentrations of AMP (0.05 and 0.1 mM), there was a delayed reaction of DTNB with the enzyme, but the titration of six SH groups (not completely shown in the figure) was ultimately obtained. At 0.5 mM AMP, less than two groups were titrated. In the presence of both AMP (0.05 mM) and FDP (0.1 mM), the pattern observed was similar to that obtained in the presence of 0.5 mM AMP alone. Thus, low concentrations of substrate appear to enhance the effectiveness of AMP. At even lower concentrations of AMP (0.001 mM or less), there was a delay in the titration, but this could not be modified by the presence of (0.01 mM) FDP. Four SH groups were ultimately titrated as in the presence of 0.1 mM FDP alone. EDTA (0.5 mM) reversed the synergistic effect of FDP on the inhibition of SH titration by AMP (Figure 4).

Effect of FDP on the Activation of FDPase by Disulfides. The synergistic effect of FDP and AMP on the activation of the enzyme was studied using 2-hydroxethyl disulfide. The enzyme was activated approximately threefold by the disulfide. The addition of AMP (0.5 mM) inhibited the activation 30%, but FDP (0.1 mM) had no effect. A combination of AMP (0.5 mM) and FDP (0.1 mM) inhibited the activation even more effectively than AMP alone (Figure 5).

Effect of Alkaline Phosphatase. After prolonged incubation of FDPase with DTNB and AMP (0.5 mM), alkaline phosphatase was added to convert the available AMP into adenosine. During the 3-hr period after the addition of alkaline phosphatase, four more SH groups became available for titration. This suggested that the four newly exposed groups belong to the set of six groups which were titrated in the absence of AMP. Phosphatase had no effect on the number of groups reactive in the absence of AMP.

TABLE II: Influence of AMP on the Number of SH Groups Reacting with Iodoacetamide and ^{14}C-Labeled PMB.

Reagent	Time (min)	SH Groups Titrated/Mole of Enzyme: Minus AMP	SH Groups Titrated/Mole of Enzyme: 0.5 mM AMP
Iodoacetamide[a]	15	6.16	4.35
	60	11.45	7.88
^{14}C-Labeled PMB[b]	120	19.40	18.00

[a] Reaction with iodoacetamide. The reaction mixture (1.0 ml) contained 100 μmoles of triethanolamine–HCl buffer (pH 7.5), 6 mμmoles of enzyme, and 12 μmoles of iodoacetamide. When AMP was included, it was added before iodoacetamide. After incubation the reaction mixture was dialyzed for 48 hr against two changes of 4 l. of water, lyophilized, and hydrolyzed in constant-boiling HCl for 22 hr at 110°. Amino acid analysis was carried out as described in the text. [b] Reaction with ^{14}C-labeled PMB. The reaction mixture (1.10 ml) contained 100 mμmoles of Tris–HCl (pH 7.5), 3.0 mμmoles of enzyme, and, where indicated, 0.5 mμmole of AMP. ^{14}C-labeled PMB (120 mμmoles, 264 cpm/mμmole) was then added. After 2 hr (Pontremoli *et al.*, 1965a), the reaction mixtures were analyzed for protein and radioactivity as described in the text.

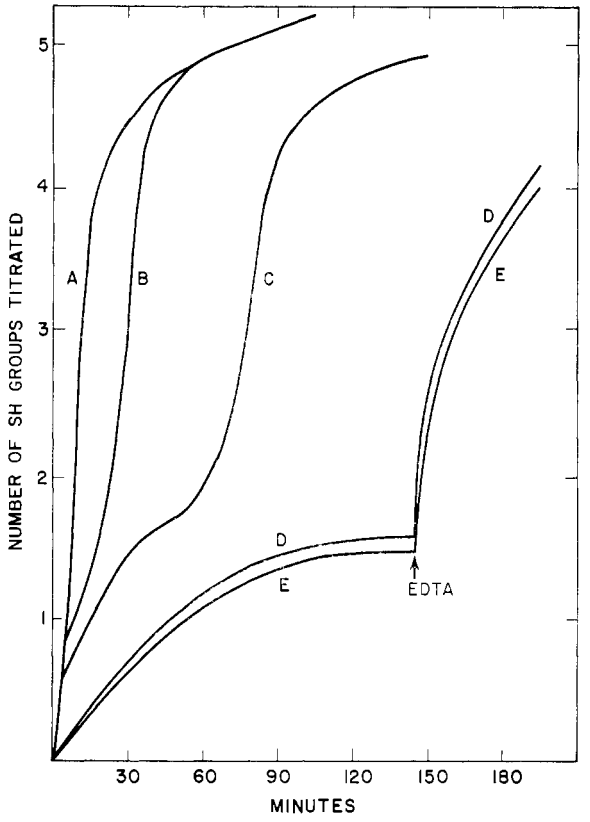


FIGURE 4: Synergistic effect of FDP and AMP on the titratability of SH groups. The conditions of the reaction are described in footnote *a*, Table I. (A) Titration carried out in the absence of added AMP. (B) Titration carried out in the presence of 0.05 mM AMP. (C) Titration carried out in the presence of 0.1 mM AMP. (D) Titration in the presence of 0.5 mM AMP. (E) Titration in the presence of 0.1 mM AMP and 0.1 mM FDP. A pattern identical with that in E was obtained in the presence of 0.05 mM AMP and AMP 0.1 mM FDP. EDTA (0.5 μmole) was added at the time indicated by the arrow.

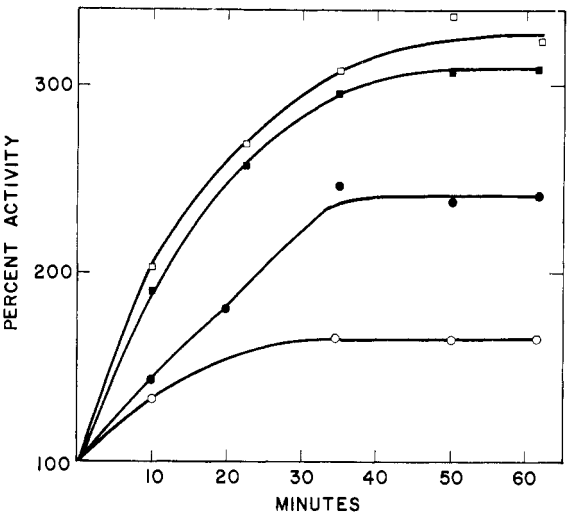


FIGURE 5: Activation of FDPase by 2-hydroxyethyl disulfide and its inhibition by AMP plus FDP. The reaction mixture (0.3 ml) at pH 7.5 contained 3 mμmoles of enzyme, 0.3 μmole of 2-hydroxyethyl disulfide, AMP, and FDP. The enzyme was assayed as described in the text. (■), Activation in the absence of added ligands; (□) activation in the presence of 0.1 mM FDP; (●) activation in the presence of 0.5 mM AMP; (○) activation in the presence of AMP (0.5 mM) and FDP (0.1 mM).

Effect of EDTA on the Inhibition of FDPase by AMP. Since EDTA reversed the effect of AMP on the titration of SH groups, the sensitivity of the enzyme to inhibition by AMP was studied in the presence of EDTA. In the presence of EDTA there was a twofold increase in the activity of the enzyme. Sensitivity to inhibition was not significantly affected.

Sensitivity of the Enzyme Treated with DTNB to Inhibition by AMP. When the enzyme has six of its SH groups titrated with DTNB (in the absence of AMP), catalytic activity was increased threefold. With only two groups titrated in the presence of 0.5 mM AMP, there was no change in the activity of the enzyme. The sensitivity to inhibition by AMP was tested in these two derivatives of the enzyme. There was no significant alteration in their sensitivity to inhibition by AMP.

Discussion

Current theories on the behavior of allosteric enzymes predict that they exist in different conformational states in the presence or absence of modifiers (Monod *et al.*, 1965; Koshland *et al.*, 1966). Evidence for conformational changes accompanying oxygen binding to hemoglobin has been reviewed by Antonini (1967) and for other systems by Atkinson (1966). Different conformational states of aspartic transcarbamylase in the presence of succinate and carbamyl phosphate or in the presence of CTP have been detected by studying the reactivity of SH groups toward PMB (Gerhart and Schachman, 1968). The data presented in this paper suggest that FDPase exists in an altered state in the presence of AMP. Only two SH groups are titrated by DTNB in the presence of AMP, in contrast to six in its absence. When 2,2′-dithiodipyridine is employed, the observed differences are even more pronounced. The differences are not so marked, however, when either PMB or iodoacetamide is employed. PMB is too reactive to distinguish between SH groups varying slightly in their reactivities. It is of interest that PMB reacted with the SH groups of both oxy- and deoxyhemoglobin, whereas iodoacetamide could react with the SH groups of oxyhemoglobin but not with those of deoxyhemoglobin (Benesch and Benesch, 1962). Similarly, PMB reacted with 11 SH groups of glyceraldehyde 3-phosphate dehydrogenase (Koeppe *et al.*, 1956) whereas tetrathionate could react with only three groups (Pihl and Lange, 1962).

The observation that the activation of the enzyme by disulfides is inhibited by AMP lends further support to the suggestion that the enzyme exists in an altered state in the presence of AMP. Of the small molecules tested, only AMP and dAMP have marked effects on the titratability of SH groups. FDP acts synergistically with AMP to decrease titratable SH groups and to diminish activation by disulfides. The data of Watanabe *et al.* (1968) reveal that AMP can bind to FDPase in the absence of substrate, although to a much smaller extent. In our experiments, concentrations of about 0.01 mM AMP were required to demonstrate the synergistic effect of FDP.

Preliminary evidence suggests that there are eight subunits in FDPase (C. L. Sia, personal communication). One set of four subunits may bind four AMP molecules. Since the

number of SH groups titrated by DTNB in the presence of AMP is reduced by four, it may be argued that AMP preferentially blocks one group in each of these chains. This decrease may be related, however, to the reactivity of the reagent employed, since the number of groups titrated by 2,2′-dithiodipyridine in the presence of AMP is decreased by about 13.

The influence of EDTA on the reversal of the effects of AMP is difficult to explain from the present data. The fact that EDTA activates the enzyme suggests that it binds to the enzyme. It does not, however, displace AMP since AMP can still inhibit the enzyme in its presence. In the presence of EDTA, the enzyme may exist in a conformational state different from that observed in the presence of AMP alone. This state may be more stable than the latter since EDTA can easily reverse the effects of AMP on SH titration.

When six SH groups were modified using DTNB (in the absence of AMP) the enzyme was activated threefold. With two groups modified (in the presence of AMP), there was no activation. The groups involved in activation are apparently masked in the presence of AMP.

Since optical rotatory dispersion patterns obtained in the range 600–280 mμ in the presence or absence of AMP did not reveal any obvious differences, and since the presence of AMP did not significantly influence the rate of digestion of FDPase by carboxypeptidase A (G. J. S. Rao, unpublished data), the changes observed by SH titration are probably minor alterations restricted to small regions of the enzyme.

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