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Conformational States of Rabbit Liver Fructose 1,6-Diphosphatase*

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SUMMARY

An analysis of the conformational states of fructose 1,6-diphosphatase by the circular dichroism technique has been performed. It has been shown that the enzyme presents a strong rigidity of its over-all conformation. The addition of either the substrate or the allosteric inhibitor, AMP, or changes in the pH of the medium, do not produce significant modifications of the secondary and tertiary structure of the protein. The same situation holds even in 0.05 M sodium dodecyl sulfate, a condition in which the quaternary structure of the enzyme undergoes a profound modification.

Small conformational changes have been detected, however, after the addition of the substrate and of AMP. These changes appear to be restricted to a limited number of tyrosyl and, at maximum, to a few vicinal residues. It is concluded that the catalytic and regulatory functioning of the enzyme do not require substantial alterations in the secondary and tertiary structure of the protein, but are related to changes in the ionization of amino acid residues and to subtle variations of the quaternary structure.

One of the central problems of modern enzyme chemistry is the definition of the role of the protein and active site conformation in catalysis.

The study of the circular dichroism of enzymes in the presence and absence of substrates and inhibitors is a well established approach to this problem.

Fructose 1,6-diphosphatase from rabbit liver constitutes a particularly interesting enzyme in this respect because of the variety of situations encountered and of the information which has been progressively accumulated on the properties of this enzyme. The enzyme requires Mg^{++} or Mn^{++} for activity (1), is active at neutral and alkaline pH (2), and is allosterically inhibited by AMP (3–5). It possesses four binding sites for the substrate and an equal number for the allosteric effector, AMP (6–8). It binds four equivalents of Mn^{++} at neutral pH and four additional equivalents above pH 8.5 (9). The enzyme, of molecular weight of 130,000, is dissociated in sodium dodecyl sulfate or by treatment with maleic anhidride into two pairs of subunits of molecular weight 29,000 to 31,000 and 35,000 to 39,000, respectively (10). It has also been shown that the binding of the substrate and of the inhibitor induces significant changes in the state of ionization of a limited number of tyrosyl residues (11) and on the reactivity of the thiol groups (12).

The present paper describes data on the circular dichroism spectra in the far and in the near ultraviolet of rabbit liver fructose 1,6-diphosphatase in the native state and in the presence of the substrate and the inhibitor AMP under various experimental conditions.

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MATERIALS AND METHODS

Rabbit liver fructose 1,6-diphosphatase was purified according to the procedure of Pontremoli *et al.* (13). Protein concentration was calculated from the absorbance at 280 mμ; a solution containing 1 mg per ml yielded an absorbance of 0.83.

D-Fructose 1,6-diphosphate sodium salt and adenosine 5′-monophosphate were obtained from Sigma. Sodium dodecyl sulfate was purchased from Schuschardt, Gorlitz, Germany. All other chemicals were reagent grade products.

Circular dichroism measurements were performed with a Roussel-Jouan CD 185 dichrograph. Samples of the protein were examined at concentrations from 0.4 to 0.9 mg per ml. Cylindrical quartz cells were used with 1-, 0.1-, 0.05-, and 0.01-cm optical path. All measurements were made at 22°. The absence of optical artifacts was determined by recording circular dichroism spectra of the same sample in cells of different optical path lengths. The data are expressed in terms of $[\theta]_\lambda$, the mean residue molecular ellipticity, defined as $[\theta]_\lambda = 3300\ (\epsilon_L - \epsilon_R)$ deg cm² dmole^{-1}, where $(\epsilon_L - \epsilon_R)$ is the difference between the molar (on a mean residue basis) extinction coefficients for left and right circularly polarized light.

RESULTS

Effect of pH and Sodium Dodecyl Sulfate on Circular Dichroism of Fructose 1,6-Diphosphatase—In Fig. 1 are reported the spectra of circular dichroism, in the near and far ultraviolet, of the native enzyme at pH 6.0, 7.5, and 9.1.

In the peptide absorption region the circular dichroism curves, in the range of pH tested, are essentially comparable except for a slight difference (if any) in the intensity of the bands. The spectra with negative extrema at 220 and 209 mμ are typical of

significant amount of β-helical structure. On assuming the absence of contributing β structures, it can be calculated that the amplitude of the bands is consistent with 40 to 50% of alpha-helical form (14). In the aromatic region (Fig. 1), the circular dichroism spectra show no substantial dependence on the pH. The curves are characterized by multiple, strongly overlapping Cotton effects. Owing to the absence of tryptophyl residues in fructose 1,6-diphosphatase, the spectrum in this zone should be assigned to optically active transitions which involve mainly tyrosyl residues. As it is seen, the conformational differences are subtle and leave essentially unaltered the backbone and side chain conformation of the protein. The circular dichroism spectra of fructose 1,6-diphosphatase in 0.05 M sodium dodecyl sulfate (Fig. 1) show that the far ultraviolet dichroic patterns of the enzyme are essentially unaffected by the presence of the detergent. Because there is indication that sodium dodecyl sulfate eliminates the β form present in proteins (15–17), the essential invariability of the circular dichroism spectra of fructose 1,6-diphosphatase after exposure to sodium dodecyl sulfate supports the idea that the beta form does not significantly contribute to the structure of the native enzyme. It can be concluded also that the process of disaggregation of fructose 1,6-diphosphatase into two subunits induced by sodium dodecyl sulfate (10) has little effect on the secondary structure of the enzyme.

Effect of Substrate on Circular Dichroism of Fructose 1,6-Diphosphatase—Fig. 2 elicits the influence of the substrate on the circular dichroism spectra of fructose 1,6-diphosphatase at pH 7.5 and 9.1. Again in the amide absorption region no substantial differences were observed, thereby emphasizing the poor conformational flexibility of the polypeptide backbone. Actually, the curves are all comparable either in the presence or absence of the substrate at both pH values.

On the contrary, the spectra in the aromatic region clearly show that the binding of the substrate induces a conformational

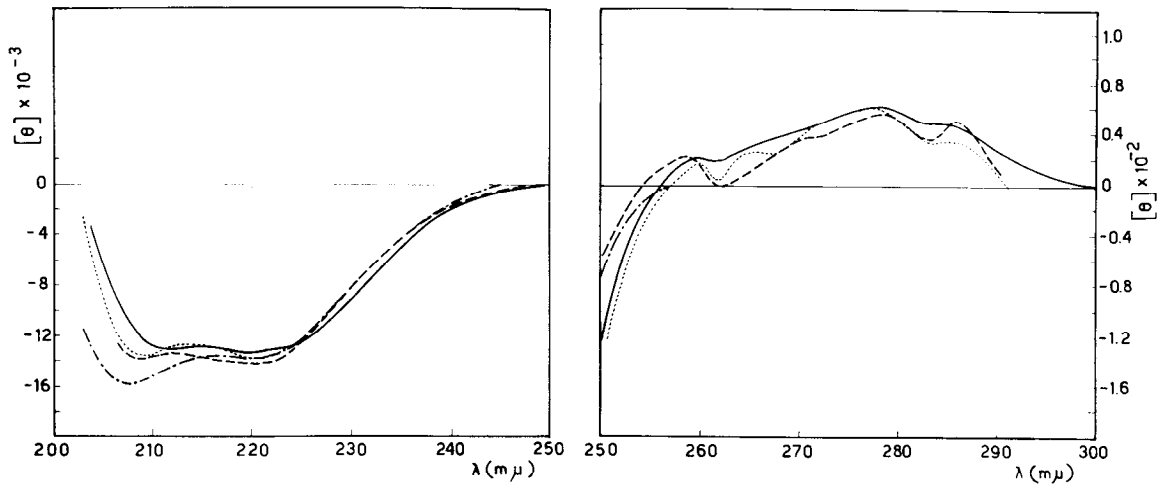

FIG. 1. Near ultraviolet (*right*) and far ultraviolet (*left*) circular dichroism spectra of fructose 1,6-diphosphatase (0.5 mg per ml) at different pH. ····, water, pH 6.0; ——, 0.02 M Tris-acetate buffer, pH 7.5; – – –, 0.02 M Tris-acetate buffer, pH 9.1; –·–·–, 0.05 M sodium dodecyl sulfate, pH 7.2. In all of the experiments the addition of divalent ions was omitted so as to eliminate catalysis.

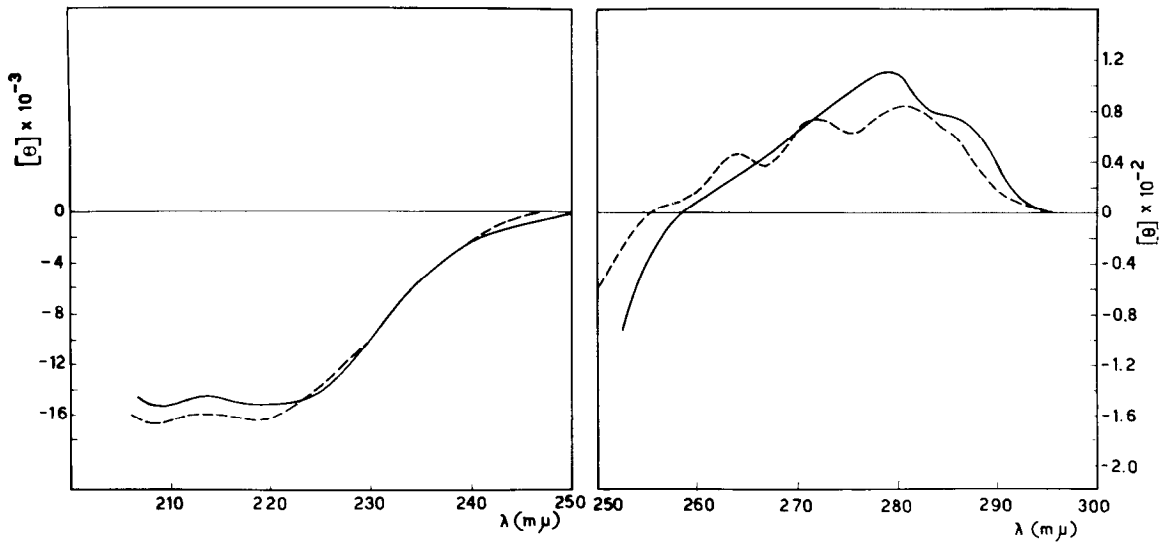

FIG. 2. Near ultraviolet (*right*) and far ultraviolet (*left*) circular dichroism spectra of fructose 1,6-diphosphatase (0.4 mg per ml) in the presence of 0.1 mM fructose 1,6-diphosphate. ——, 0.02 M Tris-acetate buffer, pH 7.5; – – –, 0.02 M Tris-acetate buffer, pH 9.1. In the presence of this concentration of the substrate, at both pH values, the binding sites of the enzyme are practically all saturated (6).

change in the state of the tyrosyl residues contributing to the optical activity. Both at pH 7.5 and 9.1, in the presence of the substrate, the positive dichroism between 260 and 285 mμ is enhanced by a significant and reproducible amount. This finding points out that fructose 1,6-diphosphatase is in two different conformational states in the presence or in the absence of the substrate.

Effect of AMP on Circular Dichroism of Fructose 1,6-Diphosphatase—The circular dichroism spectra of the enzyme in the presence of AMP are shown in Fig. 3. It is to be noted that AMP at concentration of 0.1 mM exhibits negative dichroism in the near ultraviolet in the region between 240 and 280 mμ (see inset in Fig. 3). We also present the spectra of the protein in this region obtained by substracting the contribution of AMP to the resultant dichroism. This procedure, although widely used, is to some extent not completely correct, as it is based on the assumption that the dichroic absorption of AMP will be unchanged by the binding with the protein. However, the effect of the binding on a small molecule such as AMP should presumably be small and largely overcome by an analogous effect on the protein. Therefore, the applied procedure, although *a priori* not rigorously quantitative, can be accepted in qualitative terms.

In Fig. 3 it is seen that at pH 9.1 the spectra of the protein after the binding of AMP retain only the positive band at about 287 mμ. This band is also present in the protein independently of the presence of AMP. The spectra at lower wave lengths have lost any positive dichroism and are characterized by large nega-

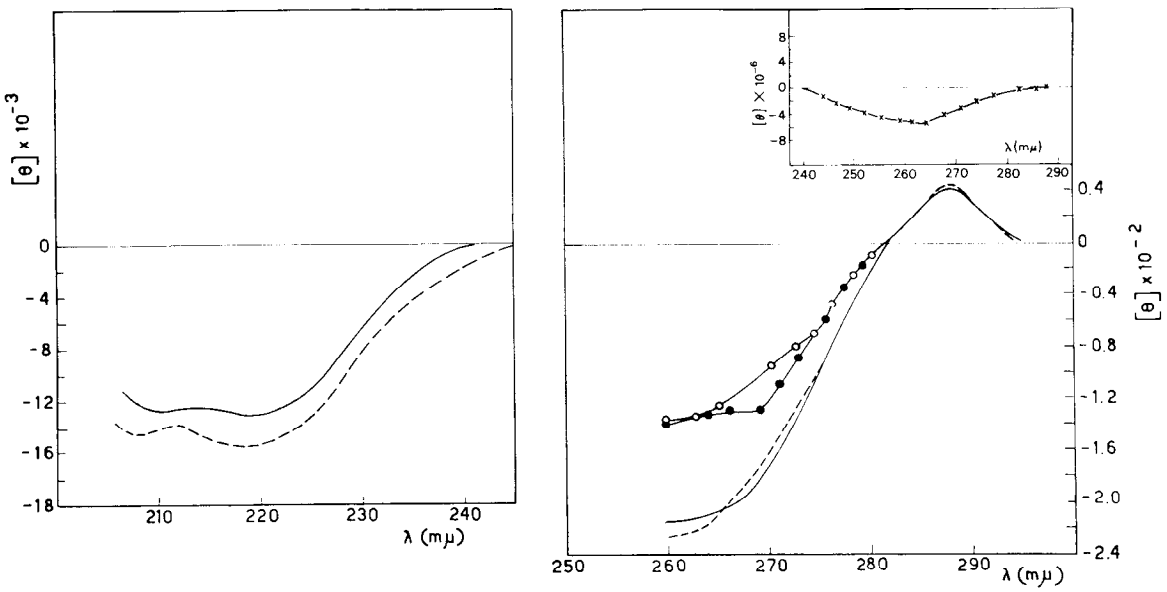

FIG. 3. Near ultraviolet (*right*) and far ultraviolet (*left*) circular dichroism spectra of fructose 1,6-diphosphatase (0.4 mg per ml) in the presence of 0.1 mM AMP. ——, 0.02 M Tris-acetate buffer, pH 7.5; – – –, 0.02 M Tris-acetate buffer, pH 9.1. ●——● and ○——○, curves at pH 7.5 and 9.1, respectively, calculated by subtracting the contribution of AMP. In the presence of this concentration of the inhibitor, at both pH values, the binding sites of the enzyme are practically all saturated (7).

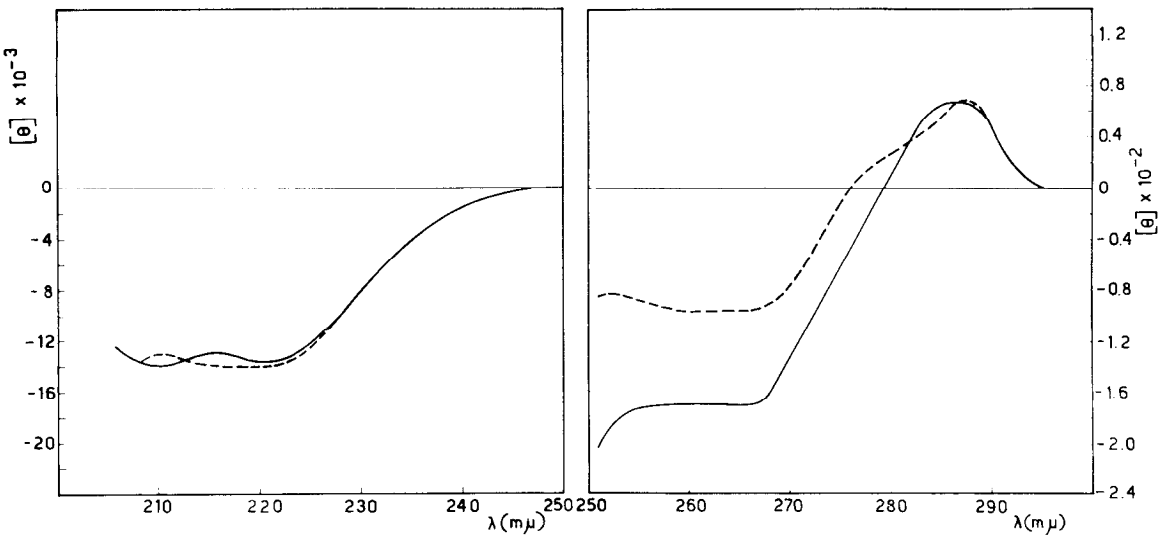

FIG. 4. Far ultraviolet (*right*) and near ultraviolet (*left*) circular dichroism spectra of fructose 1,6-diphosphatase (0.4 mg per ml) in the presence of 0.1 mM fructose 1,6-diphosphate and 0.1 mM AMP. ——, 0.02 M Tris-acetate buffer, pH 7.5; – – –, 0.02 M Tris-acetate buffer, pH 9.1. Only the curves calculated by subtracting the contribution of AMP are reported.

tive optical activity which, in our opinion, could hardly be assigned only to the contribution of changes in the optical activity of AMP itself when bound to the enzyme. The far ultraviolet circular dichroism spectra of Fig. 3 (no dichroic absorption is detectable in AMP in this region) show only small variations in the over-all conformation of the enzyme when bound to AMP both at pH 7.5 and 9.1. Indeed, a slight decrease in negative ellipticity of the bands is the only detectable effect.

Effects of Both Substrate and AMP on Circular Dichroism of Fructose 1,6-Diphosphatase—An inspection of the circular dichroism curves of fructose 1,6-diphosphatase in the presence of both fructose 1,6-diphosphate and AMP (Fig. 4) reveals that both in the far and in the near ultraviolet region the effect of the inhibitor apparently is far larger than that of the substrate. Actually, the spectra are very similar, if not identical, to those obtained in the presence of AMP alone. Therefore, it appears that the synergistic effects so far observed between the substrate and the inhibitor by means of kinetic and equilibrium studies (7, 12, 18) do not derive, within the limits of the circular dichroism technique, from a conformational alternation induced by fructose 1,6-diphosphate on the AMP-protein complex.

DISCUSSION

The data obtained in this investigation suggest a strong rigidity of the over-all conformation of fructose 1,6-diphosphatase. In fact, under conditions (0.05 M sodium dodecyl sulfate) in which the quaternary structure undergoes a profound modification, no significant changes of the secondary structure can be detected from the analysis of the circular dichroism spectra in the far ultraviolet. The same rigidity is revealed under all of the other experimental conditions tested (pH changes, addition of the substrate, and addition of AMP). However, from the inspection of the near ultraviolet circular dichroism spectra, slightly altered conformational states are revealed when the substrate or AMP is added or when the protein has been exposed to sodium dodecyl sulfate treatment.

The addition of the substrate enhances the optical activity by about 270 to 285 mμ, the effect being larger at pH 7.5 than at pH 9.1. This finding can be explained on the assumption that the substrate, on interacting with the protein, could reduce either in a direct or in an indirect way the rotational mobility of the optically active tyrosine side chains, thereby enhancing the dichroism of these aromatic chromophores. An alternative explanation could postulate that the binding of the substrate could, again directly or indirectly, render optically active some new tyrosyl residues previously inactive. Because the addition of the substrate does not influence the shape of the circular dichroism curve but only the ellipticity value, it seems more likely that the effect is related to the modification of the microenvironment of the same tyrosyl residues contributing to the optical activity of the native enzyme in the absence of the substrate.

The addition of AMP, as in the case of the substrate, affects the optical activity of the tyrosyl residues by a conformational change which has to be restricted to a limited number of tyrosyl residues and, at maximum, to a few vicinal residues, as it is shown by the invariability of the far ultraviolet circular dichroism spectra.

Because the effects of fructose 1,6-diphosphate and AMP on the enzyme markedly differ (Figs. 2 and 3), we can conclude, in accordance with previous data (11, 18), that different tyrosyl residues are affected by the binding of fructose 1,6-diphosphate and of AMP.

From our study it seems clear that the catalytic and the regulatory functioning of the enzyme, in relation to the pH, or to the presence of the substrate and of the inhibitor, do not require substantial alterations in the secondary and tertiary structure of the protein, but are related only to changes in the ionization of amino acid residues and to subtle variations of the quaternary structure.

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