UV DIFFERENCE SPECTROSCOPIC STUDIES OF THE INTERACTION OF ALLOSTERIC LIGANDS AND NEUROSPORA PYRUVATE KINASE

M. D. O'BRIEN and M. KAPOOR
Department of Biology, The University, Calgary, Alberta, Canada, T2N 1N4

Received 29 August 1975

1. Introduction

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) has been widely recognized as an important regulatory enzyme in a variety of organisms [1,2]. In previous reports on pyruvate kinase of *Neurospora,* this enzyme has been shown to be subject to regulation by a number of positive and negative allosteric modifiers [3]. The occurrence of conformational changes induced by substrates and the allosteric effector, FDP, has been demonstrated by differential proteolysis [4], response towards protein denaturants [5], studies of intrinsic protein fluorescence [4] and interaction with the hydrophobic fluorescent probe, ANS [6,7].

Investigations into the interaction of *Neurospora* pyruvate kinase and FDP demonstrated a marked reduction in the tryptophanyl fluorescence and a displacement of a substantial fraction of bound ANS [4,6] suggesting the implication of aromatic residues and nonpolar amino acid side chains in the regulatory region. Pyruvate kinase, a tetramer [3], contains a total of 3 tryptophan residues per monomer, of which two are available for modification by *N*-bromosuccinimide in the native state (submitted paper). The nonequivalence of the environment of individual tryptophans, thus indicated, can be exploited to gain further insight into the nature of the structural changes triggered by FDP.

In this work we have examined the consequence of binding of FDP to pyruvate kinase using the sensitive technique of UV difference spectroscopy. A pronounced perturbation of the environment of aromatic residues was observed.

Abbreviations: FDP, fructose 1,6-diphosphate; ANS, 8-anilino-1-naphthalene sulfonate.

2. Materials and methods

Pyruvate kinase was isolated from a wild-type strain (FGSC No. 262) of *Neurospora crassa* and purified to homogeneity, stored and assayed as described earlier [8]. Protein determinations were conducted according to the methods of Lowry et al. [9] and Babul and Stellwagen [10], with fatty-acids-free bovine serum albumin as a standard. Just prior to use the protein solutions were diluted with 0.1 M phosphate buffer, pH 7.5, dialyzed against 200 vol of the same buffer for 2 h and clarified by centrifugation at 27 000 *g* for 15 min.

UV difference spectra were measured employing a Cary Model 15 spectrophotometer. The photomultiplier dynode and sensitivity settings were 2 and 3, respectively. A set of matched, rectangular, quartz tandem cells (Hellma) with light path of 0.437 cm were used. The arrangement of compartments in the cells was as follows: compartments 1 and 2 were placed in the sample beam, 1 being closest to the monochromator; compartments 3 and 4 were in the reference beam. Compartments 2 and 4 always contained protein samples; 1 and 3 contained the reference solutions. Difference spectra were recorded from 250 nm to 340 nm on a full scale of 0.1 absorbance unit, at room temperature. Aliquots of the desired ligand were added sequentially to compart-

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ments 2 and 3 with Hamilton microsyringes, identical volumes of the buffer being added to compartments 1 and 4 at the same time.

3. Results

The difference spectra induced by the binding of FDP to *Neurospora* pyruvate kinase are presented in fig.1. These spectra show two clearly distinguishable minima located at 294 nm and 286.5 nm, a third less well-defined one in the vicinity of 277 nm and maxima at 279 and 272 nm. The two major negative peaks exhibited an increase in amplitude in the presence of increasing concentrations of FDP; the third peak also appeared to follow the same general trend albeit in a weak manner. At saturating levels of FDP, $\Delta\epsilon$ values of −6450 and −2700 were calculated for the 294 nm and 286.5 nm peaks, respectively.

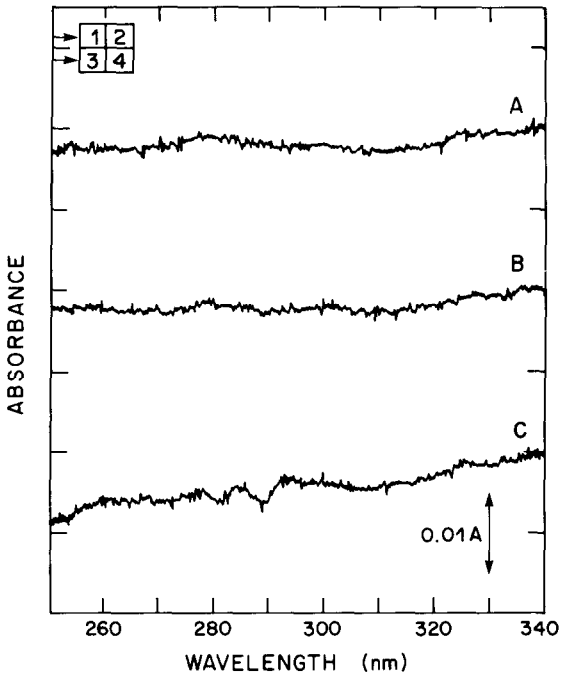

Fig.1. The difference spectra caused by the binding of FDP to pyruvate kinase. Compartments 1 and 3 contained 1.0 ml of 0.1 M phosphate buffer, pH 7.5, and compartments 2 and 4 contained 1.0 ml of pyruvate kinase solution at a concentration of 2.26 mg/ml for the baseline spectrum. For the ligand-induced spectra, aliquots of a stock solution of FDP were added to compartments 2 and 3; similar aliquots of buffer were added to compartments 1 and 4. Spectrum A, baseline. For spectra B, C and D, final concentrations of FDP were: 9.9 μM (B); 19.6 μM (C); and 47.6 μM (D).

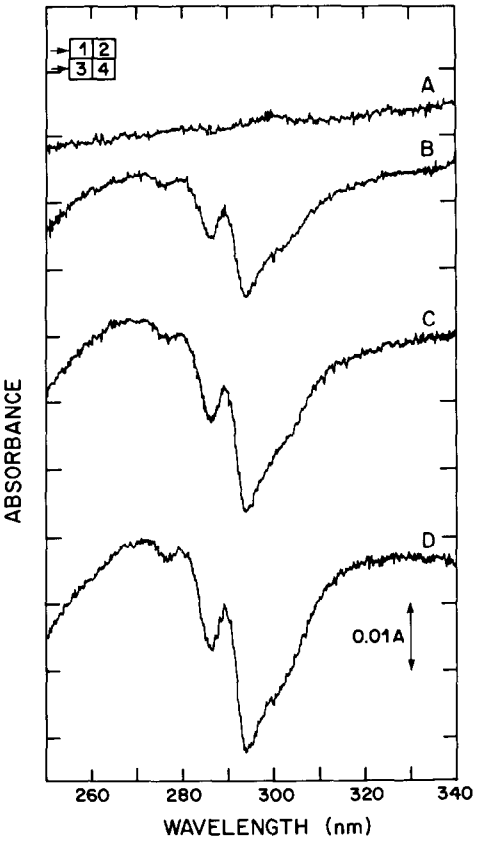

Fig.2. The difference spectra caused by the sequential addition of valine to pyruvate kinase. For the baseline spectrum (A) 1.0 ml pyruvate kinase (1.51 mg/ml) was added to compartments 2 and 4 and 1.0 ml of 0.1 M phosphate buffer, pH 7.5, to compartments 1 and 3. For ligand-promoted spectra, valine was added to a final concentration of 9.9 μM and 0.476 mM for spectra B and C, respectively. Corresponding volumes of buffer solution were added to compartments 1 and 4 each time.

The spectral effects mentioned above are characteristic of perturbations in the environment of aromatic residues. The minimum at the longer wavelength can be ascribed to perturbation of tryptophan but that at 286.5 nm could be due either to tryptophan or tyrosine, or due to contributions from both; it is difficult to distinguish unambiguously between the two at this wavelength [11,12]. The third, inconspic-

uous, peak at 276–277 nm is likely to be generated by a perturbation of tyrosyl residues. The origin of the positive peak in the neighbourhood of 270 nm is not certain.

Pyruvate kinase has previously been shown to be regulated by valine, a potent inhibitor of the enzyme [3]. Therefore, the effect of valine on the UV absorbance properties of this enzyme was also examined by difference spectroscopy. As illustrated in fig.2 valine, upon binding to the enzyme did not give rise to a well-resolved difference spectrum comparable to that produced by FDP. At saturation (Spectrum C), two barely discernible minima centered at 290 nm and 282 nm were observable. This suggests that only a weak perturbation of aromatic residues, which are affected by FDP, is generated by valine. As the concentrations of valine and FDP employed were commensurate with levels appropriate for their regulatory function, the existence of separate binding sites for these two ligands is indicated.

4. Discussion

The results documented in this communication present evidence for a perturbation in the environment of aromatic residues as a consequence of binding of the allosteric effector, FDP, to pyruvate kinase. Studies of intrinsic fluorescence and interaction with ANS indicated the implication of tryptophan residues in the FDP binding region and the presence of apolar side chains at or near this site [7]. The previously reported quenching of aromatic fluorescence, subsequent to the binding of FDP, can be explained on the basis of the positioning of specific quenching groups in their proximity or a material alteration in their environment, resulting from a ligand-induced conformational change.

The difference spectra produced by FDP are consistent with the location of aromatic residues in the FDP binding center. Changes resembling those observed with FDP would be expected if the environment of the aromatic groups were to experience a change in polarity or electrostatic charge [12]. Evidence for the propagation of discrete conformational changes by FDP has emerged using several different approaches [4–7]. Judging by the fluorescence emission maximum λ of around 350 nm, most of the tryptophan residues appear to be localized on the surface of pyruvate kinase. Therefore, even a small conformational change would be sufficient to cause a marked perturbation.

The binding of FDP to pyruvate kinase also results in an increase in the enzyme's negative charge. It is important to obtain an estimate of the distances between bound FDP, aromatic residues and other charged groups in the allosteric sites, for if these perturbations were attributable to an altered electrostatic enviroment the aromatic residues should be very close to the charged groups involved [12]. Structural changes associated with the binding of substrates, Mg^{2+} and phenylalanine, an inhibitor, have been recorded for muscle pyruvate kinase by difference spectroscopy [13–15].

Acknowledgements

We are grateful to Dr R. S. Roche for helpful discussions and the use of the Cary 15 spectrophotometer. This work was supported by an operating grant (A2565) from the National Research Council of Canada.

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