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Circular dichroism and Fourier transform infrared spectroscopic studies on the secondary structure of *Saccharomyces cerevisiae* and *Escherichia coli* phospho*enol*pyruvate carboxykinases

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Abstract

The secondary structure of *Saccharomyces cerevisiae* and *Escherichia coli* phospho*enol*pyruvate (PEP) carboxykinases was quantitatively examined using circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopies. From CD analyses, values of 24% α-helix and 38% β-sheet were obtained for the *E. coli* enzyme, while the corresponding values for the *S. cerevisiae* PEP carboxykinase were 20% and 36%. Analysis of the amide I′ infrared band indicated 20% α-helix and 36% β-sheet for the *S. cerevisiae* enzyme, while for the *E. coli* protein values of 40% β-sheet and between 9 and 36% α-helix could be inferred. It is concluded that the bacterial enzyme has more secondary structure elements than the yeast protein. No alteration of the CD or FTIR spectra was detected upon substrate or metal ion binding to any enzyme.

Keywords: Phospho*enol*pyruvate carboxykinase; Circular dichroism; FTIR; Secondary structure; (*S. cerevisiae*); (*E. coli*)

1. Introduction

Phospho*enol*pyruvate (PEP) carboxykinase [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32/49] is a key enzyme of carbohydrate metabolism, catalyzing the interconversion of PEP and oxaloacetate as shown in Eq. (1):

$$\mathrm{PEP} + \mathrm{NDP} + \mathrm{CO_2} \overset{\mathrm{Mn^{2+}}}{\rightleftarrows} \mathrm{Oxaloacetate} + \mathrm{NTP} \quad (1)$$

PEP carboxykinases isolated from microorganisms and plants are specific for adenosine nucleotides, while those from animals are specific for guanosine or inosine nucleotides [1–4]. Significant amino-acid sequence identities exists within each group of enzymes, but not between groups, except for short phosphoryl binding consensus sequences [5,6]. The enzymes from microorganisms constitute potential therapeutic targets because inhibitors specifically designed against these ATP-dependent carboxykinases may not interfere with the host's GTP-dependent enzyme [7].

Structural information can provide insights with regard to enzyme function and design of specific inhibitors. The complete tertiary structure of a protein is currently accessible by X-ray crystallography and a few closely related diffraction techniques. All these techniques require that the protein can form a well-ordered crystalline array, which may not be obtainable for some of them. The use of two-dimensional NMR spectroscopy offers a viable alternative to diffraction techniques, but is limited to low molecular weight proteins. However, CD and FTIR spectroscopies provide a valuable, global picture of the secondary structure of proteins in solution. These two spectro-

Abbreviations: CCA, Convex constraint analysis; CD, Circular dichroism; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; FTIR, Fourier transform infrared; NDT, Nucleoside diphosphate; NTP, nucleoside triphosphate; PEP, phospho*enol*pyruvate.

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scopic methods are complementary techniques because of their different, but overlapping sensitivities for various types of secondary structures. FTIR is specially suited to detect strands, while CD generally provides very good prediction of α-helix [8,9].

PEP carboxykinases from *Escherichia coli* and *Saccharomyces cerevisiae* are the only carboxykinases crystallized sofar [10,11]; however, no structural information is yet available. In view of the lack of information on important structural aspects of PEP carboxykinases, we decided to use CD and FTIR spectroscopies to analyze the secondary structure of the bacterial and yeast enzymes, taking them as models of the ATP-dependent PEP carboxykinases. Our results indicate that the bacterial enzyme has more secondary structure elements than the yeast protein.

2. Materials and methods

2.1. Enzymes

E. coli and *S. cerevisiae* PEP carboxykinases were purified as described [2,4]. Enzyme concentrations are referred to the actual concentration of the enzyme subunits, using the published molecular weights and extinction coefficients at 280 nm [2,12,13].

2.2. Circular dichroism spectra

CD spectra were recorded in a Jasco J-720 spectropolarimeter calibrated with (+)-10-camphorsulfonic acid. Ultraviolet spectra in the 195–260 nm range were determined in 0.10 mm cells at 25°C. Enzymes (1.5 mg/ml) were dissolved in 10 mM potassium phosphate buffer (pH 7.4). Spectra were acquired at a scan rate of 20 nm/min, and the average of 5 scans was recorded. The mean residue ellipticity [Θ], was calculated (from ellipticity readings) using a mean residue molecular weight of 111. No changes were detected when the spectra were acquired from solutions in 10 mM Hepes buffer (pH 7.4). To estimate the content of secondary structure, the CD data were analyzed by the CCA algorithm, that operates on a collection of spectral data in the 195 to 240 nm wavelength range to extract the common spectral components with their spectral weights [14,15], and also by the method of Yang et al. [16].

2.3. Infrared spectroscopy

FTIR spectra were collected with a Nicolet 5PC FTIR spectrometer interfaced with an IBM compatible computer operating Nicolet data collection software. The spectrometer was equipped with a liquid nitrogen cooled mercury/cadmium telluride detector. The sample chamber was purged constantly with dry, CO_2-depleted air. Lyophilized enzyme samples were dissolved to a final concentration of 1.1 mM in 50 mM Hepes buffer (pD 7.5) and introduced into a sample cell fitted with CaF_2 windows and a 25 μm Teflon spacer. A time-dependent shifting of the amide I′ band to lower frequencies, concomitant with a slight narrowing of the band for spectra taken soon after sample dissolution was indicative of deuterium/hydrogen exchange. The exchange was complete after six hours, and only spectra taken six or more hours after sample dissolution were used for structural analysis. Spectra were recorded at 20°C at a resolution of 2 cm^{-1} by collecting 512 co-added interferograms, apodizing with a Happ–Genzel window, before applying the Fourier transform. In order to compensate for absorption

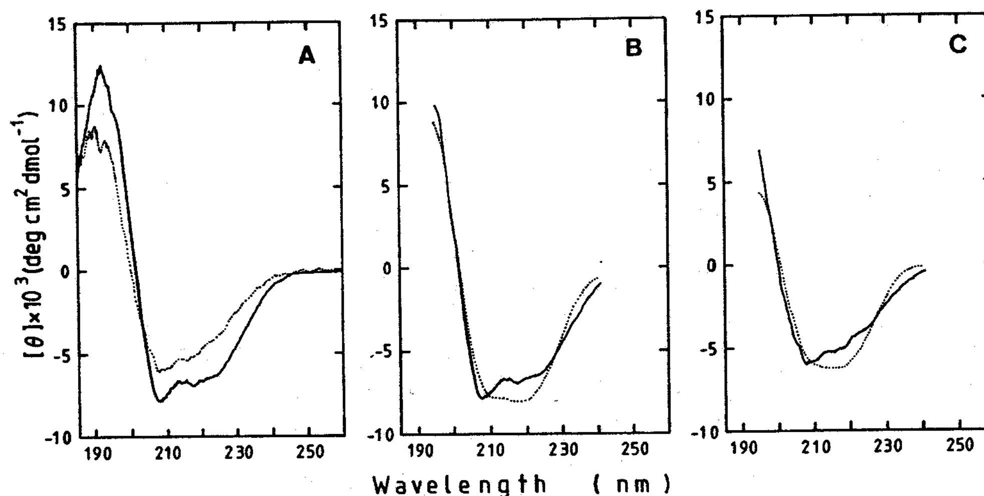

Fig. 1. Far-UV CD spectra of PEP carboxykinases. In (A), the unsmoothed *S. cerevisiae* (· · ·) and *E. coli* (—) PEP carboxykinases spectra are shown. Spectra were run in duplicate and data averaged. The experimental spectra (—) for the *E. coli* (B) and *S. cerevisiae* (C) enzymes are compared with the respective reconstructed spectrum (· · ·) in the 195–240 nm range.

due to traces of water in the sample, a spectrum of buffer acquired under identical conditions was subtracted from each sample. In addition, absorption due to traces of water was compensated by substraction of a spectrum of vapor. A flat, featureless baseline between 1700 and 1800 cm^{-1} was the criterion for the adequacy of spectral adjustments [17]. Data files were transferred to a VAX work station for analysis. Programs used for resolution enhancement [18] and non-linear least-squares fitting of the Gaussian bands to the enhanced spectra were similar to those described by others [17]. Analysis was carried out on the IDL platform (Research Systems) using routines kindly provided by Dr. Meir Shinnar, Department of Radiology, University of Pennsylvania.

3. Results

3.1. Circular dichroic spectroscopy

Fig. 1 shows the CD spectra of the two enzymes in the 185–260 nm range. In this region, both spectra show positive peaks around 192 nm and negative peaks at 208 nm, while the *E. coli* enzyme presents a negative shoulder around 224 nm. Analysis of the spectra by means of the CCA algorithm using a four component system [14,15] gave the structural coefficients shown in Table 1. The comparison of the pure components obtained from each CD spectrum allowed a clear assignment for three of them to α-helix, β-sheet, and random type described by Perczel et al. [14]; however, the fourth component could not be assigned to β-turns or other secondary structure component. The use of a five-component system did not allow a better assignment (not shown). The comparison between each spectrum and the respective back-calculated spectrum based on a four component system using the CCA algorithm is shown in Fig. 1. Analysis of the CD spectra by the method of Yang et al. [16] gave a poorer fit (not shown) with 11% α-helix, 66% β-sheet, and 23% unordered structure content for the *S. cerevisiae* enzyme, and 20% α-helix, 55% β-sheet, 22 unordered, and 3% β-turn structure for the *E. coli* PEP carboxykinase. Saturating concentrations of $MnCl_2$, ATP, PEP, MnATP, MgATP or $NaHCO_3$ did not alter the recorded spectra (not shown). The reproducibility in the analysis of the CD spectra in terms of percentage of secondary structure within each method was approx. ±1%, that is, the last figure given.

Table 1
Deconvolution of *S. cerevisiae* and *E. coli* PEP carboxykinases CD spectra by the convex constraint analysis method into four components

Enzyme	First (α-helix)	Second (β-sheet)	Third (unordered)	Fourth (other)
S. cerevisiae	20	36	14	30
E. coli	24	38	16	22

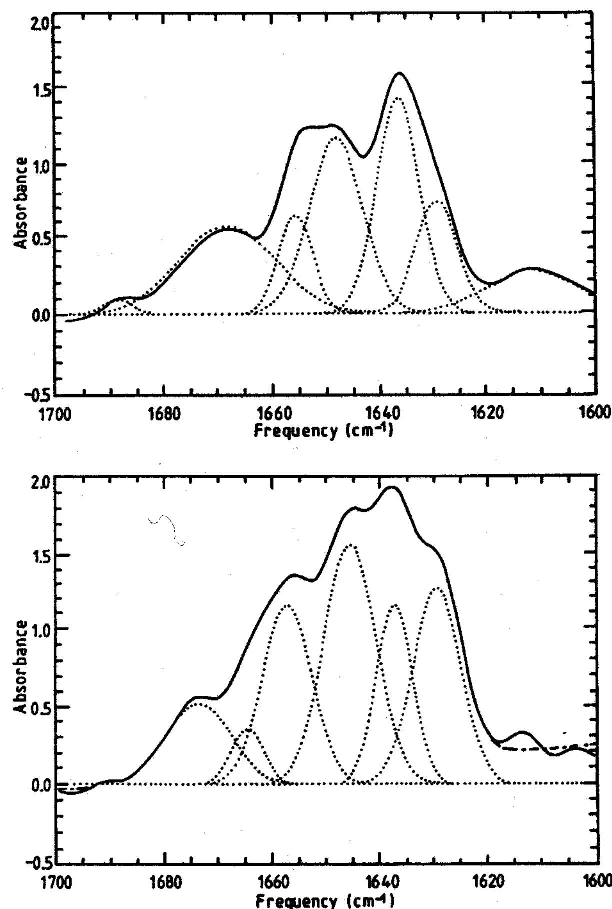

Fig. 2. Curve-fitted deconvoluted spectra of PEP carboxykinases (pD 7.5). Top panel, *E. coli*; bottom panel, *S. cerevisiae*. (—) Experimental data, (· · ·) are the Gaussian fitted bands, and (– · –) the sum of the fitted bands. (The dash-dotted line is obscured due to the high quality of the fit.)

3.2. Fourier transform infrared spectroscopy

Fig. 2 shows the curve-fitted spectra of *S. cerevisiae* and *E. coli* PEP carboxykinases in the amide I′ region. In order to check the band decomposition procedure, the amide I′ contour was reconstituted by addition of the component bands, showing almost superimposable spectra. Peak maxima positions, bandwidths at half height, and percentages of band areas are shown in Table 2. The assignment of the individual bands to secondary structure elements is based on FTIR data of proteins published recently [8,19,20]. The band at 1648 cm^{-1} for the *E. coli* spectrum is in a region usually assigned to unordered structure [21,22]. However, the fact that for some proteins it has been shown that α-helices absorb below 1650 cm^{-1} [8,20], and also our CD spectroscopy results that indicate 24% α-helix for this enzyme, suggest that this absorption peak may be due to non-resolved peaks originated from unordered plus α-helix structures. No spectral changes were observed upon addition of saturating concentrations

Table 2
Frequencies (ν), bandwiths at half-height ($\Delta\nu_{1/2}$), fractional band areas, and structure assignments for *S. cerevisiae* and *E. coli* PEP carboxykinases

ν (cm^{-1})	$\Delta\nu_{1/2}$ (cm^{-1})	A (%)	Assignment
S. cerevisiase			
1629	10	21	β
1637	8	15	β
1645	11	28	unordered
1657	10	20	α
1664	7	4	turns
1674	12	12	turns
E. coli			
1629	8	13	β
1637	9	27	β
1648	11	27	α + unordered
1656	7	9	α
1668	20	23	turns
1688	7	1	turns

of substrates or metal ions to any enzyme (not shown). The reproducibility of data acquired for different samples was correct within 1%.

4. Discussion

An important assumption of methods using Fourier deconvolution and then band-fitting or other quantitation procedures [17,19,23,24] is that the integrated molar absorptions are equal for each type of structure. This assumption is considered valid owing to the agreement between experimental FTIR data and that derived from X-ray analysis of many proteins [25]. By summing up the band areas assigned to particular secondary structure elements, a content of 20% α-helix, 36% β-sheet, 16% turns, and 28% unordered structures was calculated from the FTIR data for the *S. cerevisiae* PEP carboxykinase. For the *E. coli* enzyme, the FTIR data indicate 40% β-sheet and 24% turns, while the amount of α-helix shown in the 1656 cm^{-1} band appears somewhat low (9%). However, there are cases where α-helix elements give rise to absorption bands below 1650 cm^{-1} [8,20], in a region where absorption due to unordered structures is expected. For this reason and considering the CD data, the most likely possibility is that the 1648 cm^{-1} absorption band arises from both α-helix and unordered structures. Based on these arguments, α-helix would comprise between 9 and 36% of the protein for the *E. coli* enzyme, while the unordered structures would be less than 27%.

Several methods can be used for secondary structure determination using protein CD spectra. The helical content can be estimated directly from molar ellipticity at 222 nm, whereas analysis of protein structure in terms of the proportion of α-helix, β-sheet, β-turn, and other structures can be obtained by decomposing the measured CD spectrum in terms of a set of basis spectra representing the four individual secondary structures [9]. However, it is well known that deconvolution of circular dichroism spectra, because of its inherent limitations and assumptions [16,26–28] is a procedure which gives secondary structure estimations which should not be treated as accurate absolute values, but are extremely useful to detect changes [29]. In the present case the values of secondary structure content are shown to depend on the method employed (see Section 3, Table 1). However, the enzyme from *E. coli* contains more α-helix ($6 \pm 2\%$) than the enzyme from yeast, irrespective of the method of analysis employed. The fact that the percents of α-helix and β-strand estimated for the yeast enzyme by the CD and FTIR methods are coincident suggests that these values may be reasonable estimates of the average secondary structure of this PEP carboxykinase. For the α-helix content there is very good agreement between these two methods for the *S. cerevisiae* enzyme (20%), while the amount estimated by FTIR from the 1656 cm^{-1} for the *E. coli* PEP carboxykinase is lower than that determined from the CD experiments. However, considering the arguments given in the first paragraph, the amount of α-helix determined by FTIR may be underestimated. Hence, we believe that the α-helix content given by CD represents a better measure of this particular secondary structure element for this enzyme.

The observation from both spectroscopic methods that these two microorganism PEP carboxykinases are highly structured (i.e., more than 55/60% α-helix or β-strand) is consistent with their resistance to trypsin attack [13,30]. Dill and co-workers [31,32], on the basis of the theory for polymer chains, have predicted that helices, sheets, and turns should increase with the compactness of the molecule. Preliminary inspection of the predicted secondary structures of the *E. coli* and *S. cerevisiae* PEP carboxykinases did not permit assignment of the difference in the average secondary structure content to the extra extension of the yeast enzyme. The secondary structure percentages derived from the two spectroscopic techniques do not agree well with the predominant α-helix and low β-strand content predicted for both enzymes by secondary structure prediction algorithms based on amino-acid sequence. For example, the recently described method of Rost and Sander [33] predicts 26–27% α-helix and 24–21% β-sheet for *E. coli* and *S. cerevisiae* PEP carboxykinases, respectively. Discrepancies with predictive secondary structure methods have been reported before [22,34].

The lack of alteration on the CD or FTIR spectra of these two carboxykinases upon substrate binding indicates that the changes in the intrinsic fluorescence previously detected upon Mn^{2+} or MgATP binding to the *E. coli* enzyme [4] or upon CO_2 binding to the *S. cerevisiae* carboxykinase [30], are due to changes in the local microenvironment of tryptophanyl residues, and do not involve major changes in the secondary structure of the proteins. For the yeast PEP carboxykinase, it has been

proposed that the decrease in the intrinsic fluorescence of the protein that follows CO_2 binding may be due to changes of the microenvironment around Trp-88 and Trp-89 [30]. There are proteins where relatively large changes in the secondary structure are produced upon the addition of substrates, inhibitors, or metal ions. For example, by using FTIR, Arrondo et al. [35] have reported that ATP or P^1, P^5-di(adenosine-5′)pentaphosphate (a competitive inhibitor against ATP) binding produce a decrease in the content of β-strands and an increase in the amount of turns on *E. coli* adenylate kinase. Similarly, significant differences in the secondary structure of the Ca^{2+}-bound (holo) and the Ca^{2+}-free (apo) forms of α-lactalbumin have been detected by Prestrelski and Thompson [36] by the same technique.

In conclusion, using two complementary spectroscopic techniques we have analyzed the secondary structure of *S. cerevisiae* and *E. coli* PEP carboxykinases, two proteins that share 42% amino-acid identity. The results indicate that the bacterial enzyme has more secondary structure elements than the yeast carboxykinase, and that their global secondary structure does not change upon substrate or metal ion binding.

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References

[1] Utter, M.F. and Kolenbrander, H.M. (1992) The Enzymes, 3rd Ed., Vol. 6, Academic Press, New York, pp. 117–168.
[2] Medina, V., Pontarollo, R., Glaeske, D., Table, H. and Goldie, H. (1990) J. Bacteriol. 172, 7151–7156.
[3] Weldon, S.L., Rando, A., Matathias, A.S., Hod, Y., Kalonick, P.A., Savon, S., Cook, J.S. and Hanson, R.W. (1990) J. Biol. Chem. 265, 7308–7317.
[4] Encinas, M.V., Rojas, M.C., Goldie, H. and Cardemil, E. (1993) Biochim. Biophys. Acta 162, 195–202.
[5] Traut, T.W. (1994) Eur. J. Biochem. 222, 9–19.
[6] Bossemeyer, D. (1994) Trends Biochem. Sci. 19, 201–205.
[7] Linss, J., Goldenberg, S., Urbina, J.A. and Amzel, L.M. (1993) Gene 136, 69–77.
[8] Surewicz, W.K, Mantsch, H.H. and Chapman, D. (1993) Biochemistry 32, 389–394.
[9] Johnson, W.C. (1988) Annu. Rev. Biophys. Biophys. Chem. 17, 145–166.
[10] Delbaere, L.T.J., Vandonselar, M., Glaeske, D., Jabs, C. and Goldie, H. (1991) J. Mol. Biol. 219, 593–594.
[11] Jacob, L.R., Vollert, H., Rose, M., Entian, K.-D., Bartunik, L.J. and Bartunik, H.D. (1992) J. Chromatogr. 625, 47–54.
[12] Cardemil, E., Encinas, M.V. and Jabalquinto, A.M. (1990) Biochim. Biophys. Acta 1040, 71–76.
[13] Goldie, H. and Sanwall, B.D. (1980) J. Biol. Chem. 255, 1399–1405.
[14] Perczel, A., Hollósi, M., Tusnády, G. and Fasman, G.D. (1991) Protein Eng. 4, 669–679.
[15] Perczel, A., Park, K. and Fasman, G.D. (1992) Anal. Biochem. 208, 83–93.
[16] Yang, J.T., Wu, C.S.C. and Martínez, M.M. (1986) Methods Enzymol. 130, 208–259.
[17] Byler, D.M. and Susi, H. (1986) Biopolymers 25, 469–487.
[18] Kauppinen, J.K., Moffat, D.J., Mantsch, H.H. and Cameron, D.G. (1981) Appl. Spectrosc. 35, 271–276.
[19] Surewicz, W.K. and Mantsch, H.H. (1988) Biochim. Biophys. Acta 952, 115–130.
[20] Naumann, D., Schultz, C., Grneö-Tschelnokow, D. and Hucho, F. (1993) Biochemistry 32, 3162–3168.
[21] Fabian, H., Naumann, D., Misselwitz, R., Ristau, O., Gerlach, D. and Welfle, H. (1992) Biochemistry 31, 6532–6538.
[22] Kralicek, A.V., Vesper, N.A., Ralston, G.B., Wake, R.G. and King, G.F. (1993) Biochemistry 32, 10216–10223.
[23] Dong, A., Huan, D. and Caughey, W.S. (1990) Biochemistry 29, 9185–9193.
[24] Lee, D.C., Haris, P.I., Chapman, D. and Mitchell, R.C. (1990) Biochemistry 29, 9185–9193.
[25] Haris, P.I. and Chapman, D. (1992) Trends Biochem. Sci. 17, 328–333.
[26] Manavalan, P. and Johnson, W.C., Jr. (1983) Nature, 305, 831–832.
[27] Woody, R.W. (1978) Biopolymers 17, 1452–1457.
[28] Sreerama, A. and Woody, R.W. (1994) J. Mol. Biol. 242, 497–507.
[29] Andreu, J.M., De la Torre, J. and Carrascosa, J.L. (1986) Biochemistry 25, 5230–5239.
[30] Herrera, L., Encinas, M.V., Jabalquinto, A.M. and Cardemil, E. (1993) J. Protein Chem. 12, 413–418.
[31] Dill, K.A. and Shortle, D. (1991) Annu. Rev. Biochem. 60, 795–825.
[32] Chan, H.S. and Dill, K.A. (1989) Macromolecules 22, 4559.
[33] Rost, B. and Sander, C. (1993) J. Mol. Biol. 232, 584–599.
[34] Park, K., Flynn, G.C., Rothman, J.E. and Fasman, G.D. (1993) Protein Sci. 2, 325–330.
[35] Arrondo, J.L.R., Gilles, A.M., Bärzu, Fermandjian, S., Yang, P.W. and Mantsch, H.H. (1989) Biochem. Cell Biol. 67, 327–331.
[36] Prestrelski, S. and Thompson, M.P. (1991) Biochemistry 30, 8797–8804.