# A comparison of the secondary structure of human brain mitochondrial and cytosolic 'malic' enzyme investigated by Fourier-transform infrared spectroscopy

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The secondary structure of human brain cytosolic and mitochondrial 'malic' enzymes purified to homogeneity has been investigated by Fourier-transform IR spectroscopy. The absorbance IR spectra of these two isoenzymes were slightly different, but calculated secondary-structure compositions were essentially similar (38% $\alpha$-helix, 38–39% $\beta$-sheet, 14% $\beta$-turn and 9–10% random structure). These proportions were not affected by succinate, a positive effector of mitochondrial 'malic' enzyme activity. IR spectra indicate that the tertiary structures of human brain cytosolic and mitochondrial 'malic' enzymes are slightly different, and addition of succinate does not cause conformational changes to the tertiary structure of the mitochondrial enzyme. Thermal-denaturation patterns of the cytosolic and mitochondrial enzymes, obtained from spectra recorded at different temperatures in the absence or presence of $Mg^{2+}$, suggest that the tertiary structure of both isoenzymes is stabilized by bivalent cations and that the cytosolic enzyme possesses a more compact tertiary structure.

## INTRODUCTION

$NADP^+$-dependent 'malic' enzyme [L-malate dehydrogenase (oxaloacetate-decarboxylating) ($NADP^+$); EC 1.1.1.40] catalyses the bivalent-metal-dependent oxidative decarboxylation of malate using $NADP^+$ as electron acceptor to produce pyruvate and $CO_2$. In previous papers we have reported subregional and intracellular distribution [1] and purification of two isoenzymes of $NADP^+$-linked 'malic' enzyme from human brain [2]. The isoenzymes, localized in the mitochondria and cytosol, display clear differences in some of their physicochemical, catalytic, kinetic and regulatory properties [2,3]. Most striking were their different abilities to catalyse reductive carboxylation of pyruvate to malate and their allosteric properties, activation by succinate or fumarate being a property of the mitochondrial enzyme only [2,3]. The significant differences in the kinetic properties of the two isoenzymes may suggest that conformational changes occur on binding of activators (bivalent metal cations) or positive effectors (succinate or fumarate).

Loeber et al. [4] recently isolated a cDNA of cytosolic $NADP^+$-dependent 'malic' enzyme from human fat-cells. The cDNA nucleotide sequence has an open reading frame of 1719 bp coding for a protein of 572 amino acids with a predicted molecular mass of 64.1 kDa [4]. Human brain cytosolic $NADP^+$-dependent 'malic' enzyme has an identical molecular mass estimated by SDS/PAGE [2]. A cDNA of mitochondrial $NADP^+$-dependent 'malic' enzyme from human hippocampus has also been obtained [5]. The cDNA nucleotide sequence contained an open reading frame coding for a protein of 604 amino acids with a predicted molecular mass of 67 kDa [5]. After purification, the mitochondrial enzyme from human brain showed a single protein band corresponding to a size of 64 kDa on SDS/PAGE [2]. The difference between the estimated molecular mass [2] and that predicted from the cDNA sequence [5] is probably due to a cleavage signal peptide sequence (approx. 30 N-terminal amino acids). The amino acid sequence of the human brain mitochondrial $NADP^+$-dependent 'malic' enzyme showed significant similarity to the cytoplasmic enzyme (approx. 71% identity) [5].

Electron micrographs showed that cytosolic 'malic' enzyme from pigeon liver has a planar structure with four subunits arranged at the corners of a square [6]. Hsu and Pry [7] suggested a sequential model for this enzyme to explain the kinetic results on half-of-the-sites reactivity. Chang and co-workers [8,9] proposed an alternative pre-existing asymmetrical model. These two models can only be regarded as hypothetical working models, as no crystal structure of this enzyme is yet available. However, a diffraction-quality crystal of rat liver 'malic' enzyme has been successfully prepared [10].

CD spectroscopic measurements indicate that pigeon liver cytosolic 'malic' enzyme has a high degree of ordered structure [11]. The content of $\alpha$-helix was estimated to be 33%, which is consistent with the value (37%) for rat liver 'malic' enzyme predicted from the primary structure [12], whereas the $\beta$-pleated sheet was estimated to be 15% [11] or even less [12]. The markedly different secondary-structure composition of the $NAD^+$-linked 'malic' enzyme from *Ascaris suum* estimated by CD and the PROSEC program has also been reported [13].

It would therefore be of interest to investigate using Fourier-transform IR (FTIR) spectroscopy (a sensitive physical method for monitoring protein secondary structure) whether the cytosolic and mitochondrial 'malic' enzymes from human tissues have similar or different secondary and tertiary structures. The aim of this work was also to study the effect of $Mg^{2+}$, an activator of

Abbreviations used: FTIR spectroscopy, Fourier-transform IR spectroscopy; DTT, dithiothreitol.

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both enzyme forms, and succinate, which is known to be a positive effector of the mitochondrial enzyme only [3], on the secondary and tertiary structures of these isoenzymes.

We report here further characterization of human brain mitochondrial and cytosolic 'malic' enzyme, which complement the data presented recently [1–3].

## MATERIALS AND METHODS

### Chemicals

$^2H_2O$ (99.9%) and $NaO^2H$ were purchased from Aldrich. All other chemicals used were of the highest purity available and were purchased as described previously [2]. Cytosolic and mitochondrial 'malic' enzymes from human brain were prepared as described recently [2]. The purity of the proteins analysed was estimated by SDS/PAGE. 'Malic' enzyme activity was assayed as described previously [2].

### Preparation of samples for FTIR-spectroscopic measurements

Purified protein solutions were concentrated on Centricon 30 microconcentrators (Amicon Division, W. R. Grace, Danvers, MA, U.S.A.) and washed several times with 10 mM Hepes/$NaO^2H$/1 mM dithiothreitol (DTT)/1 mM EDTA buffer prepared in $^2H_2O$, $p^2H$ 7.2 ($p^2H$ = pH-meter reading + 0.4) [14]; Centricon 30 microconcentrators were also used for the washing procedure. After being washed, the protein was concentrated to 4% (w/v). The concentrated protein solution (prepared in the above buffer with or without 2.5 mM $MgSO_4$) was stored for 3 days at 4 °C.

### FTIR spectra

The concentrated protein solutions were placed in a thermostatically controlled Specac 20500 cell (Specac Ltd., Orpington, Kent, U.K.) fitted with $CaF_2$ windows, using 12 $\mu$m spacers. The cell was maintained at 20 °C (results presented in Figures 1 and 2) or at the temperature indicated in Figures 4 and 5, using an external bath circulator (HAAKE F3). Spectra were recorded by means of a Perkin–Elmer Cetus Instruments 1760-x FTIR-spectrometer. Typically, 256 scans at 2 $cm^{-1}$ resolution were averaged for each sample; a normal Beer–Norton apodization function and a deuterated triglycine sulphate detector were used in these experiments. During data acquisition, the spectrometer was continuously purged with dry air at a dew point of −40 °C. Spectra of the buffer were acquired under the same scanning and temperature conditions. An interactive difference routine was used to subtract the spectrum of the buffer from that of the sample. Appropriate subtraction of water spectra was judged to yield an approximately flat baseline at 1900–1480 $cm^{-1}$ [15]. Subtraction of $^2H_2O$ spectra was adjusted to the removal of the $^2H$-O-$^2H$ bending absorption near 1220 $cm^{-1}$ [15]. Second-derivative spectra were calculated over a nine-data-point range (9 $cm^{-1}$). Spectral deconvolution was performed using the Perkin–Elmer ENHANCE function, which is analogous to the method developed by Kauppinen et al. [16]. The deconvolution parameters for the amide I′ band were set with the half-bandwidth at 18 $cm^{-1}$ and a resolution enhancement factor of 2.25.

### Quantitative analysis of the secondary structure by curve fitting

Amide I′ band contour of deconvoluted absorbance spectra (obtained in a $^2H_2O$ medium) was fitted with Gaussian, Lorentzian or Voigt curves by using Peakfit version 3.11 computer software (Jandel Scientific, Erkrath, Germany). The calculations were performed by the method of Blume et al. [17]. The position and the number of amide I′ components, used as an input for the curve-fitting program, were obtained from the second-derivative, fourth-derivative and deconvoluted absorption spectra. In each curve, the position, width and height were considered as free parameters, the values of which were adjusted using a recursive method. The position of the curves was allowed to vary within a range of $\pm 5$ $cm^{-1}$ with respect to the frequencies used as input. The goodness of fit was visualized by both a line obtained as the difference between the experimental values and the calculated ones and the corresponding S.D. The best fit was obtained with a Gaussian band shape. Standard errors of curve fitting were 0.0030 and 0.0036 for the cytosolic and mitochondrial enzyme respectively.

## RESULTS

FTIR spectra of human brain cytosolic and mitochondrial 'malic' enzyme at 20 °C on subtraction of spectra for buffer prepared in $^2H_2O$ are shown in Figures 1 and 2 respectively. The spectra (amide I′ band) obtained for the two isoenzymes are essentially similar and exhibit a maximum at 1646 $cm^{-1}$ (Figures 1 and 2, spectrum A). Furthermore, only slight differences between the two isoenzymes were seen in the deconvoluted (Figures 1 and 2, spectrum B) and second-derivative (Figures 1 and 2, spectrum C) spectra. The deconvoluted and second-derivative spectra of the 'malic' enzyme revealed amide I′ component bands at 1686, 1670, 1659, 1651, 1644, 1635 and 1628 $cm^{-1}$ for the cytosolic and 1688, 1680, 1668, 1657, 1650, 1644 and 1634 $cm^{-1}$ for the mitochondrial enzyme. According to

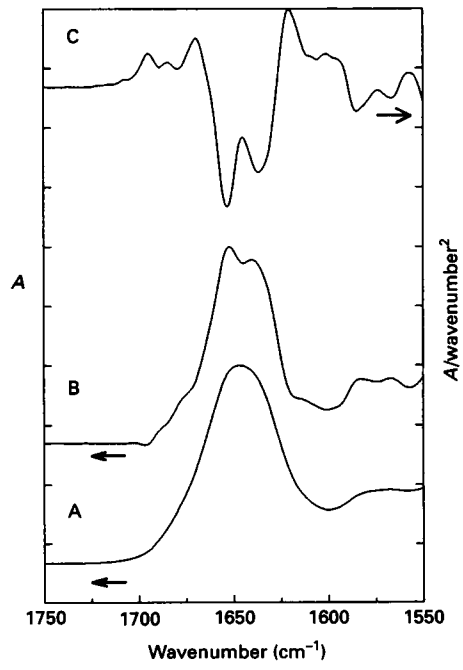


**Figure 1 FTIR spectra of cytosolic 'malic' enzyme**

Cytosolic 'malic' enzyme (approx. 40 mg of protein/ml) was suspended in $^2H_2O$ medium containing 10 mM Hepes/$NaO^2H$, $p^2H$ 7.2, 1 mM EDTA and 1 mM DTT. A, Original absorbance spectrum; B, same spectrum after band narrowing by Fourier deconvolution; C, same spectrum after resolution enhancement by second-derivative calculation over a nine-data-point range (9 $cm^{-1}$).

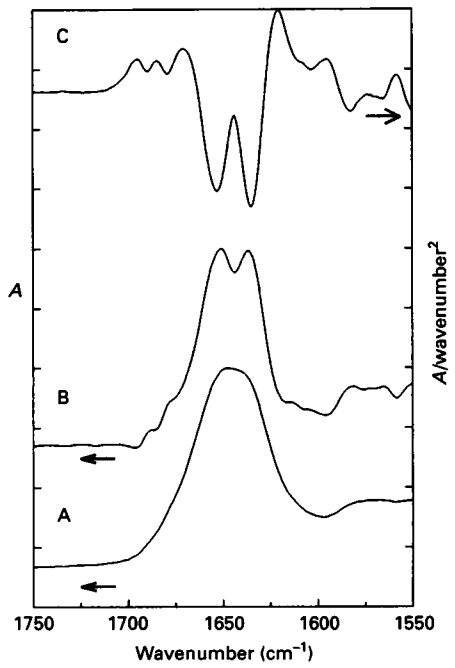


**Figure 2 FTIR spectra of mitochondrial 'malic' enzyme**

Mitochondrial 'malic' enzyme (approx. 40 mg of protein/ml) was suspended in $^2H_2O$ medium containing 10 mM Hepes/NaO$^2$H, p$^2$H 7.2, 1 mM EDTA and 1 mM DTT. A, Original absorbance spectrum; B, same spectrum after band narrowing by Fourier deconvolution; C, same spectrum after resolution enhancement by second-derivative calculation over a nine-data-point range (9 $cm^{-1}$).

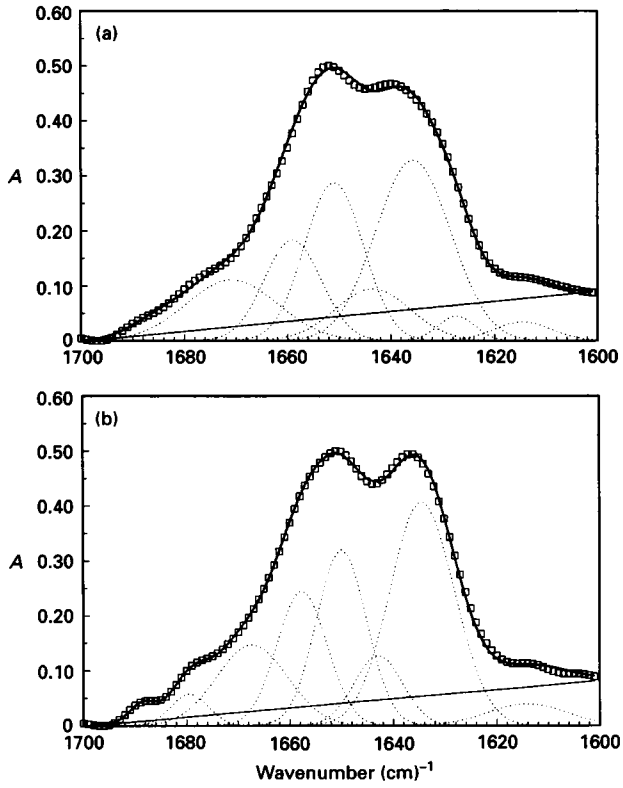


**Figure 3 Amide I′ band contour with individual components fitted for (a) cytosolic and (b) mitochondrial 'malic' enzyme**

□, Experimental data; ——, sum of individual components; · · · ·, individual component bands.

**Table 1 Positions and fractional areas of the amide I′ component bands for cytosolic and mitochondrial 'malic' enzyme from human brain**

| | Cytosolic enzyme | | Mitochondrial enzyme | |
|---|---|---|---|---|
| Structure | Peak centre ($cm^{-1}$) | Area (%) | Peak centre ($cm^{-1}$) | Area (%) |
| β-Sheet | 1686 | 2 | 1688 | 1 |
| β-Sheet | – | – | 1680 | 3 |
| β-Turn | 1670 | 14 | 1668 | 14 |
| α-Helix | 1659 | 15 | 1657 | 17 |
| α-Helix | 1651 | 23 | 1650 | 21 |
| Random | 1644 | 10 | 1644 | 9 |
| β-Sheet | 1635 | 34 | 1634 | 35 |
| β-Sheet | 1628 | 2 | 0 | 0 |

well-defined criteria [18] we assigned the band observed in $^2H_2O$ medium at 1657–1650 $cm^{-1}$ to α-helix, the bands at 1635–1628 $cm^{-1}$ to β-sheet structure, the bands at 1644 $cm^{-1}$ to random (non-ordered) structure and the bands at 1670 and 1668 $cm^{-1}$ to β-turns. The bands observed at 1688 and 1680 (for the mitochondrial enzyme) and at 1686 (for the cytosolic enzyme) are probably due to β-sheet structure [19]. Figures 3(a) and 3(b) show the curve-fitted amide I′ band contour of cytosolic and mitochondrial 'malic' enzyme respectively. The data presented in Table 1 summarize the calculated positions and fractional areas of the amide I′ component bands for cytosolic and mitochondrial 'malic' enzyme from human brain. Analysis of FTIR spectra showed that these two isoenzymes have similar secondary structures consisting of 38% α-helix, 38–39% β-sheet, 14% β-turn and 9–10% random structure.

The addition of succinate, a positive effector of the mitochondrial enzyme [3], to both isoenzymes did not induce any changes in the secondary structure (not shown). This information could also be obtained by comparing the deconvoluted spectra of cytosolic and mitochondrial 'malic' enzyme in the absence and presence of succinate. These spectra are almost identical, suggesting that no changes occurred in the secondary structure of the isoenzymes on binding a positive effector (not shown). FTIR spectra may also give indirect information on the tertiary structure of a protein. This information may be obtained from H/$^2$H exchange studies and/or from FTIR spectra recorded at different temperatures in $^2H_2O$ [20–22]. Such spectra also give information on the thermal denaturation of a protein [23]. Preincubation of mitochondrial 'malic' enzyme at different temperatures showed that the onset of enzyme denaturation occurs between 40 and 45 °C and increases gradually up to 55 °C (Figure 4). Rapid denaturation starts at 55 °C, the maximum being observed between 65 and 70 °C. A slightly different pattern was observed for the cytosolic enzyme: sharper peaks were obtained. These data suggest that the cytosolic and mitochondrial 'malic' enzymes from human brain possess slightly different tertiary structures. We observed no marked differences in the FTIR spectra of the mitochondrial enzyme at different temperatures in the presence and absence of succinate (not shown). This suggests that succinate does not cause significant changes in the tertiary structure of mitochondrial 'malic' enzyme.

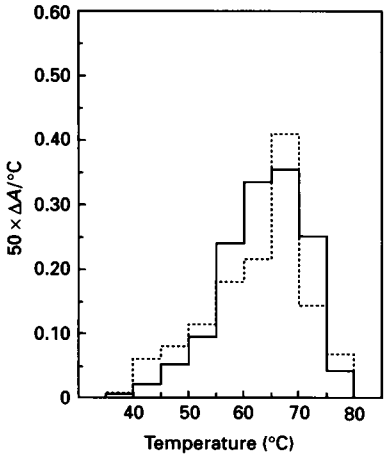


**Figure 4 Temperature-dependent changes in the $\Delta A$/°C parameter calculated for cytosolic (· · · ·) and mitochondrial (——) 'malic' enzyme**

'Malic' enzyme (approx. 40 mg of protein/ml) was suspended in $^2H_2O$ medium containing 10 mM Hepes/NaO$^2$H, p$^2$H 7.2, 2.5 mM $MgSO_4$, 1 mM EDTA and 1 mM DTT. The $\Delta A$/°C parameter was calculated after normalization of the amide I′ band spectra at 0.5A.

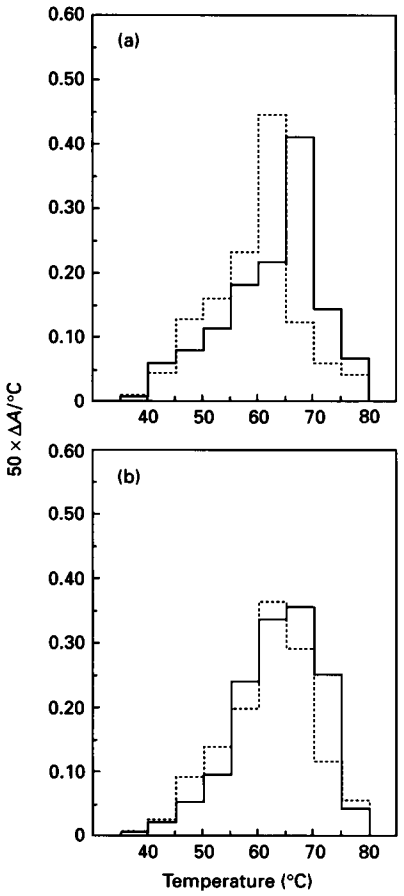


**Figure 5 Temperature-dependent changes in the $\Delta A$/°C parameter calculated for cytosolic (a) and mitochondrial (b) 'malic' enzyme in the presence and absence of $Mg^{2+}$**

'Malic' enzyme (approx. 40 mg of protein/ml) was suspended in $^2H_2O$ medium containing 10 mM Hepes/NaO$^2$H, p$^2$H 7.2, 2.5 mM $MgSO_4$, 1 mM EDTA and 1 mM DTT (——) or 10 mM Hepes/NaO$^2$H, p$^2$H 7.2, 1 mM EDTA and 1 mM DTT (· · · ·). The $\Delta A$/°C parameter was calculated after normalization of the amide I′ band spectra at 0.5A.

The regulatory properties and conformational states of 'malic' enzyme from different sources have been shown to alter in the presence of bivalent metal cations [3,5,24,25]. The deconvoluted spectra of both human brain mitochondrial and cytosolic 'malic' enzyme in the presence of $Mg^{2+}$ did not differ from those obtained in the absence of $Mg^{2+}$ (not shown). These data suggest that binding of $Mg^{2+}$ to 'malic' enzyme (either cytosolic or mitochondrial) does not induce significant changes in the secondary structures of these proteins. Figure 5(a) shows the $\Delta A$/°C parameter plotted as a function of temperature for cytosolic 'malic' enzyme in the absence and presence of $Mg^{2+}$. In the absence of $Mg^{2+}$, maximum enzyme denaturation occurred at 65 °C whereas in the presence of the cation it occurred at 70 °C. For the mitochondrial enzyme, the absorbance changes occurred within a narrower range of temperatures but the onset of denaturation was also shifted to higher temperatures in the presence of $Mg^{2+}$ (Figures 5a and 5b). These data indicate that binding of bivalent metal cation induces changes in the tertiary structure of the mitochondrial and cytosolic 'malic' enzymes making them more rigid and stable.

## DISCUSSION

The data published so far indicate several differences in some physicochemical, kinetic, catalytic and regulatory properties between human brain cytosolic and mitochondrial 'malic' enzymes [1–3]. They suggest that *in vivo* the cytosolic enzyme catalyses reductive carboxylation of pyruvate (or both reductive carboxylation of pyruvate and oxidative decarboxylation of malate) whereas the mitochondrial enzyme catalyses the decarboxylation reaction only [1–3]. Furthermore, in contrast with the cytosolic enzyme, the mitochondrial enzyme has been found to exhibit a sigmoid substrate-saturation curve at low malate concentration which was accentuated at higher pH values and in the presence of low concentrations of $Mg^{2+}$ or $Mn^{2+}$ [3]. At low malate concentration the activity of the mitochondrial 'malic' enzyme was increased considerably by succinate or fumarate [3]. These differences suggest that the two isoenzymes are probably different proteins as far as secondary and tertiary structures are concerned. Furthermore, we would have expected succinate to induce different conformational states. To test this hypothesis, we examined the effect of succinate, which is an effector of the mitochondrial enzyme only, and $Mg^{2+}$, an activator of both isoenzymes, on the structure of these proteins. To the best of our knowledge, this is the first detailed analysis of mitochondrial and cytosolic 'malic' enzymes from the same tissue using FITR spectroscopy.

Our data indicate that the two forms of human brain 'malic' enzyme have a high degree of ordered structure, i.e. a high percentage of $\alpha$-helix and $\beta$-structure. As far as $\alpha$-helix content is concerned our results are essentially similar to those reported by Lee et al. [11] and Magnuson et al. [12] for pigeon and rat liver cytosolic 'malic' enzyme respectively. However, significant differences were obtained for $\beta$-sheet content. We found 38 and 39% $\beta$-sheet content in the cytosolic and mitochondrial isoenzymes respectively, whereas that of pigeon liver (cytosolic) 'malic' enzyme was estimated to be less than 15% [11] and CD data obtained for rat liver cytosolic 'malic' enzyme indicated only 3% [12]. These discrepancies may arise from the different sensitivities of the FTIR and CD spectroscopy in detecting $\beta$-sheet and $\alpha$-helix structures. In particular, FTIR spectroscopy is

more sensitive than CD spectroscopy for the determination of β-sheet, as the characteristic bands due to this type of structure are well resolved and thus can be efficiently estimated in the curve-fitting calculation. Thus it is reasonable to believe that the secondary structures of the human brain, pigeon and rat liver (cytosolic) $NADP^+$-linked 'malic' enzymes are essentially similar. This is probably not true for *Ascaris suum* $NAD^+$-linked 'malic' enzyme. The secondary-structure composition of this enzyme analysed by CD spectroscopy was as follows: α-helix, 49%; β-sheet and β-turn, 31%; random coil, 20%. This may suggest that $NAD^+$-linked 'malic' enzyme has a different secondary structure from that of the $NADP^+$-linked enzyme.

We recently showed that activity of the mitochondrial enzyme was increased considerably by succinate and fumarate [3]. In contrast, oxidative decarboxylation catalysed by the cytosolic enzyme was not affected by either [3]. As no significant differences in the secondary structure of mitochondrial 'malic' enzyme were induced by succinate, the activating effect of this dicarboxylic acid may be due to either changes in the tertiary structure (i.e. in its conformation) or small changes in secondary structure that could not be detected by FTIR spectroscopy. Essentially similar results were reported by Rajapaksa et al. [13], who showed that the binding of malate or the malate analogue tartronate produces only a slight change in the secondary structure of *Ascaris suum* $NAD^+$-linked 'malic' enzyme analysed by CD spectroscopy.

The thermal stability of mitochondrial 'malic' enzyme was also studied by FTIR spectroscopy, as this technique allows changes that occur in each amide I′ component band to be followed as a function of temperature. An increase in temperature leads to a decrease in intensity of the α-helix and β-sheet bands, an increase in intensity of the band due to random structures and usually the appearance of two new bands at about 1680 and 1620 $cm^{-1}$. The slightly different denaturation patterns of cytosolic and mitochondrial 'malic' enzymes (Figure 4) suggest that these isoenzymes have different tertiary structures. In particular, the higher temperature at which maximum cytosolic enzyme denaturation occurs suggests that this protein possesses a more compact tertiary structure than the mitochondrial enzyme. This is in accord with the well-known fact that cytosolic 'malic' enzyme is more stable than the mitochondrial enzyme during purification and storage.

In the presence of succinate, an activator of the mitochondrial enzyme only, the denaturation patterns of the proteins studied did not change significantly (not shown). This suggests that, despite the significant effect of succinate on mitochondrial 'malic' enzyme activity, the tertiary structure of the enzyme was not affected. However, in the presence of $Mg^{2+}$, an activator of both isoenzymes of 'malic' enzyme, the denaturation of the cytosolic and mitochondrial enzymes were both altered: the onset of denaturation was shifted to higher temperatures for both isoenzymes in the presence of the bivalent metal cation. These data indicate that the binding of $Mg^{2+}$ induces changes in the structures of the cytosolic and mitochondrial enzymes making them more rigid and stable.

Taken together, the results indicate that, despite significant differences in physicochemical, catalytic, kinetic and regulatory properties, human brain cytosolic and mitochondrial 'malic' enzymes exhibit only slight differences in their secondary and tertiary structures as studied by FTIR spectroscopy. Furthermore, no structural changes were observed on addition of succinate, a known activator of the mitochondrial enzyme. The thermal denaturation patterns of cytosolic and mitochondrial 'malic' enzyme indicate that the latter has a less compact tertiary structure, which is not affected by succinate, but is changed by bivalent metal cation.

To our knowledge this is the first report of comparative studies on secondary and tertiary structures using FTIR spectroscopy of cytosolic and mitochondrial 'malic' enzyme from the same tissue.

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