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Involvement and Identification of a Tryptophanyl Residue at the Pyruvate Binding Site of Transcarboxylase†

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ABSTRACT: Transcarboxylase (TC) from *Propionibacterium shermanii* consists of a central hexameric 12S subunit to which 6 outer dimeric 5S subunits are attached through 12 biotinyl 1.3S subunits. The enzyme catalyzes the transfer of a carboxyl group from methylmalonyl-CoA to pyruvate, forming oxalacetate and propionyl-CoA. The pyruvate binding site, located on the 5S subunit, was examined by monitoring the intrinsic fluorescence quenching accompanying the incremental addition of pyruvate to either TC or the 5S subunit. The quenching studies indicate that there are two binding sites for pyruvate with apparent dissociation constants of 0.23 and 1.25 mM for intact TC and of 0.18 and 1.20 mM for the outer 5S subunit. The microenvironment of the Trp(s) sensitive to pyruvate binding was analyzed by using the neutral quencher acrylamide. With TC, the fractional accessible fluorescence (f_a) was 0.64, whereas a f_a value of 0.56 was obtained in the presence of pyruvate. A 27% decrease in f_a was observed with the outer 5S subunit in the presence of pyruvate as compared to the free 5S subunit. By labeling the outer subunit in the absence of pyruvate with 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl), a tryptic peptide containing DNPS-labeled Trp was isolated; the sequence was determined and identified with the amino-terminal residues 67-75 of the outer subunit that has been derived from DNA-sequencing studies. Trp-73 contained the DNPS label; its labeling was inhibited by pyruvate. A sequence comparison with other biotinyl enzymes shows that the sequence 67-75 is highly conserved. On the basis of these results, it is proposed that sequence 67-75 of the outer subunit may constitute a part of the pyruvate binding region of transcarboxylase.

Transcarboxylase from *Propionibacterium shermanii* consists of a central 12S subunit to which 6 outer 5S subunits are attached through 12 biotinyl 1.3S subunits [for review of the structure see references Wood and Zwolinsky (1976) and Wood and Kumar (1985)]. The biotinyl subunit has a biotin prosthetic group linked to the ϵ-NH_2 group of Lys-89 through an amide bond (Maloy et al., 1979). The enzyme catalyzes the transfer of a carboxyl group from methylmalonyl-CoA to pyruvate, forming oxalacetate and propionyl-CoA. This reaction consists of two partial reactions; the central 12S subunit catalyzes the first partial reaction in which a carboxyl group from methylmalonyl-CoA is transferred to the biotinyl subunit, forming propionyl-CoA and the carboxybiotinyl subunit. The second partial reaction is catalyzed by the outer 5S subunit, where the carboxyl group is transferred to pyruvate, forming oxalacetate, and the biotinyl subunit is regenerated (Chuang

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et al., 1975). The central 12S subunit, a hexamer (M_r 360 000), contains 12 CoA ester binding sites, two per monomer (Poto et al., 1978). The pyruvate binding sites are on the outer 5S subunit, which is a dimer of M_r 120 000 (Chuang et al., 1975). It is a metalloprotein containing 1 mol of Co^{2+} or Zn^{2+} per mol of the monomer (Ahmad et al., 1972). Also, both the metals have been shown to be located at the keto acid (pyruvate) binding sites (Northrop & Wood, 1969; Fung et al., 1974).

Intrinsic fluorescence studies have shown that the tryptophanyl fluorescence of the outer subunit is enhanced by the binding of the biotinyl subunit (Kumar & Wood, 1982) or its various functional domains (Kumar et al., 1982a,b). Furthermore, *N*-bromosuccinimide modification studies (Kumar et al., 1988) have shown that certain Trps are essential for the activity of the enzyme. These studies indicated that there may be one or more Trps present at or near the biotinyl subunit-binding domain(s) of the outer subunit and possibly at the catalytic sites of the enzyme as well. In the present study we have examined the involvement of Trps at the pyruvate binding site of the outer subunit. We have also assessed the microenvironment of Trps of both the intact transcarboxylase and the outer subunit in the absence and presence of pyruvate, using acrylamide, a neutral quencher of tryptophanyl fluorescence (Lehrer, 1971). Using the tryptophan-specific reagent 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl),[1] we have shown that Trp-73 of the outer 5S subunit of transcarboxylase is involved during pyruvate interaction.

Materials and Methods

Materials

Acrylamide, pyruvate, and 2,4-dinitrophenylsulfenyl chloride were purchased from Sigma; TPCK-trypsin was from Worthington, and trifluoroacetic acid, phenyl isothiocyanate, and dansyl chloride were from Pierce. Sequencer reagents were supplied by Applied Biosystems Inc.

Methods

Methods such as preparation of transcarboxylase and subunits, fluorescence measurements, procedure for trypsinization, and high-performance liquid chromatography separation of peptides used in this study were essentially similar to those used by Kumar et al. (1988).

Acrylamide Quenching Studies. Fluorescence intensities were determined by using an excitation wavelength of 295 nm and by continuous monitoring of the emission at 340 nm. The final intensity at any given acrylamide concentration was taken as a time-averaged value ($n = 10$). Additions of acrylamide were made from a concentrated (8.0 M) stock solution. Fluorescence was corrected for dilution and inner filter effect (Hélène et al., 1969). A correction for absorption of the acrylamide itself at 295 nm was made according to the method of McClure and Edelman (1967) using a molar extinction coefficient of 0.29 M^{-1} cm^{-1} at 295 nm. The details of fluorescence titrations of transcarboxylase and the outer 5S subunit with the substrate pyruvate are described in the figure legends.

Data Analysis. Fluorescence quenching was analyzed according to the Stern–Volmer relationship (eq 1) (Stern & Volmer, 1919), where F_0 is the fluorescence in the absence of quencher, F is the fluorescence at the molar quencher concentration [Q], and K_{SV} is the Stern–Volmer quenching constant obtained from the slope of a plot of F_0/F versus [Q]. For multifluorophore proteins, the Stern–Volmer plot will be nonlinear when the individual fluorophores are not equally accessible to the quencher. A modified Stern–Volmer equation (eq 2) has been applied by Lehrer (1971) to such heterogeneous systems. From a plot of $F_0/(F_0 - F)$ versus $[Q]^{-1}$, the values of $f_{a(eff)}$, the maximum fractional accessible fluorescence (1/intercept), and $K_{Q(eff)}$, the effective quenching constant (intercept/slope), are obtained.

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

$$F_0/F_0 - F = 1/f_{a(eff)} + 1/f_{a(eff)}K_{Q(eff)}[Q] \quad (2)$$

Table I: Physical Parameters for Pyruvate and Acrylamide Interactions with the Intact Transcarboxylase and the Outer 5S Subunit[a]

	with pyruvate		with acrylamide	
sample	max quenching (%)	K_{app} (mM)	K_Q (M^{-1})	$f_{a(eff)}$
transcarboxylase (TC)	66	0.23; 1.25	9.45	0.64
TC + pyruvate			6.73	0.56
5S subunit	50	0.18; 1.20	6.55	0.74
5S + pyruvate			5.94	0.54

[a] The experimental details are similar to the conditions described under Figures 1, 3, and 4.

Chemical Modification of Tryptophans and Isolation of DNPS-tryptophanyl Peptide of the Outer 5S Subunit. The tryptophanyl residues of the outer subunit were modified with DNPS-Cl by using the method described by Scoffone et al. (1968). After labeling, the outer subunit was washed with dry acetone followed by extensive dialysis against 100 mM ammonium bicarbonate, pH 7.8, to remove the excess reagent. The labeled outer subunit was treated with TPCK-trypsin; the tryptic digest was lyophilized and then dissolved in 0.1% TFA. The peptides were separated on a reverse-phase C-4 column using a Du Pont HPLC system. Peptides were eluted by using a linear gradient generated from 0.1% TFA/H_2O and 0.1% TFA/CH_3CN. The labeled peptides were monitored at the absorption maximum of the dinitrophenyl group, 350 nm. The labeled peptides were subsequently purified on a reverse-phase C-18 column (Synchropak).

NH_2-Terminal and Amino Acid Analysis. The amino-terminal residue of the isolated peptides was determined by the dansyl method (Bruton & Hartley, 1970). For amino acid analysis, the peptides were hydrolyzed in vacuo in 6 N HCl for 18 h. The amino acid composition was determined by a modified PTC method as described by Phillips and Wood (1986).

Sequence Determination. Sequence analyses were carried out in an Applied Biosystems Model 470A sequencer (Hunkapiller et al., 1983). Phenylthiohydantoins (PTH) were identified by complementary HPLC systems (Glajch et al., 1985; Ericsson et al., 1977). Quantitative evaluation of the PTH amino acids was as described by Smithies et al. (1971) and Machleidt and Hofner (1981).

Results

Determination of the Apparent Dissociation Constant of Pyruvate with the Intact Transcarboxylase and the Outer 5S Subunit. Addition of incremental amounts of pyruvate (up to 10 mM) caused progressive quenching of the tryptophanyl fluorescence of transcarboxylase (Figure 1). A maximum of 66% quenching was observed with ~10 mM pyruvate (Figure 1B and Table I). This fluorescence quenching was not accompanied by any detectable change in the emission maxi-

[1] Abbreviations: DNPS-Cl, 2,4-dinitrophenylsulfenyl chloride; TFA, trifluoroacetic acid; Trp, tryptophan; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; TC, transcarboxylase.

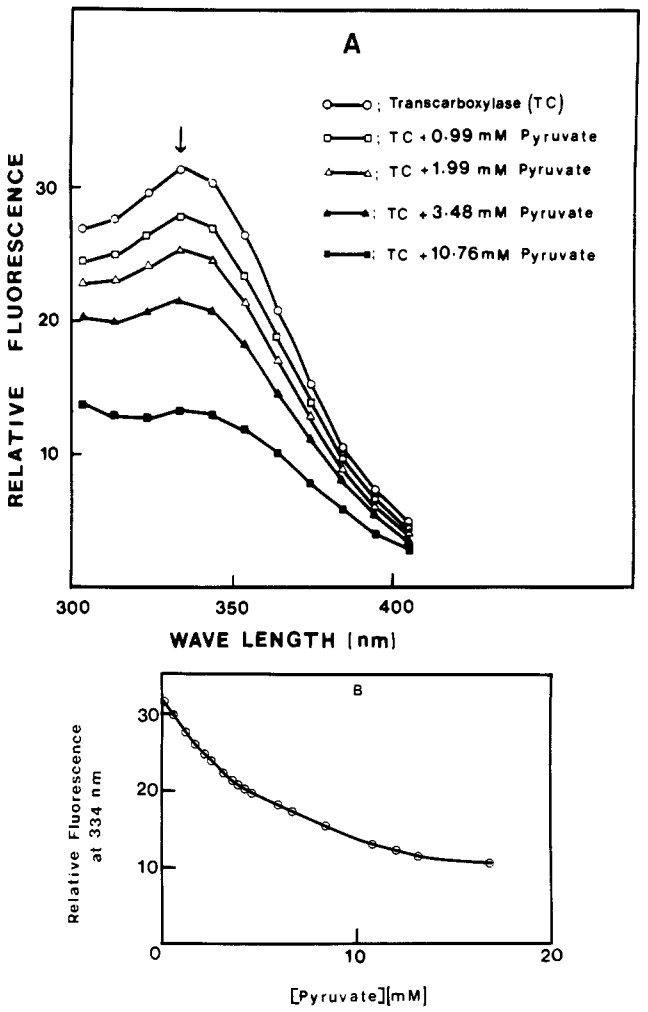

FIGURE 1: (A) Effect of pyruvate on the tryptophanyl fluorescence of transcarboxylase (TC). [TC] = 100 μg/mL; solvent, 250 mM sodium acetate, pH 5.5. Excitation is at 295 nm. Representative emission spectra are shown. (B) Plot of relative fluorescence intensity of TC at the emission maximum (334 nm) as a function of concentration of pyruvate.

mum. On the basis of the assumption that fluorescence quenching is an indirect measure of pyruvate binding to transcarboxylase and by applying the equation of McClure and Edelman (1967) to the quenching data of Figure 1B, the apparent dissociation constant (K_D) of pyruvate can be derived. As shown in Figure 2, a biphasic plot was obtained with two apparent dissociation constants, viz., 0.23 and 1.25 mM, respectively, (Table I), suggesting the existence of low- and high-affinity binding sites.

With the isolated outer 5S subunit, a fluorescence quenching similar to that of the intact enzyme was observed (data not shown). A maximum quenching of ~50% was seen at ~10 mM pyruvate. The K_D values for pyruvate with respect to the outer 5S subunit were found to be 0.18 and 1.20 mM (Table I).

Determination of the Environment of Tryptophans (Trp) of Intact Transcarboxylase (TC) both in the Absence and in the Presence of Pyruvate by Acrylamide Quenching. We have assessed changes in the microenvironment of the Trps of transcarboxylase as a result of pyruvate interaction. For this purpose, the enzyme was titrated both in the presence and in the absence of pyruvate with acrylamide, a commonly used neutral quencher of Trp fluorescence (Lehrer, 1971). Acrylamide is an effective quencher of the Trps of transcarboxylase, and as shown in Figure 3A, a Stern–Volmer plot

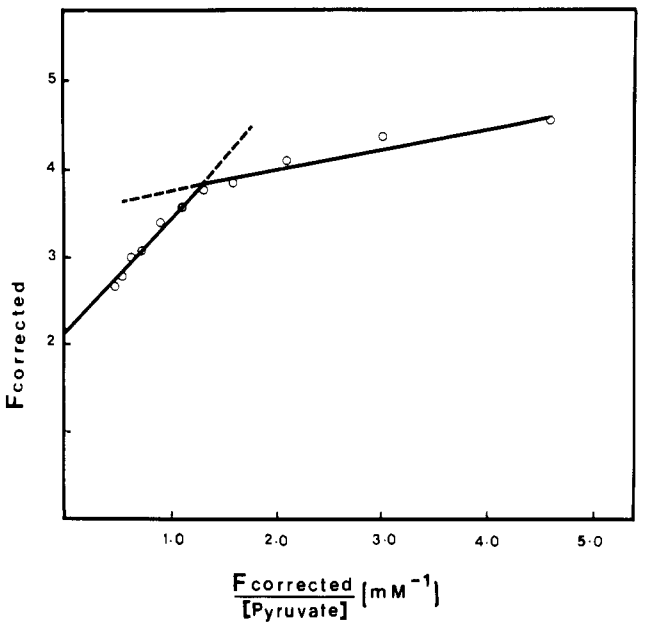

FIGURE 2: Analysis of fluorescence quenching data of transcarboxylase (from Figure 1B) as a function of pyruvate concentration using the McClure and Edelman (1967) equation. The fluorescence of transcarboxylase at each pyruvate concentration was corrected for self-absorption, inner filter effect, and dilution effects as described under Methods.

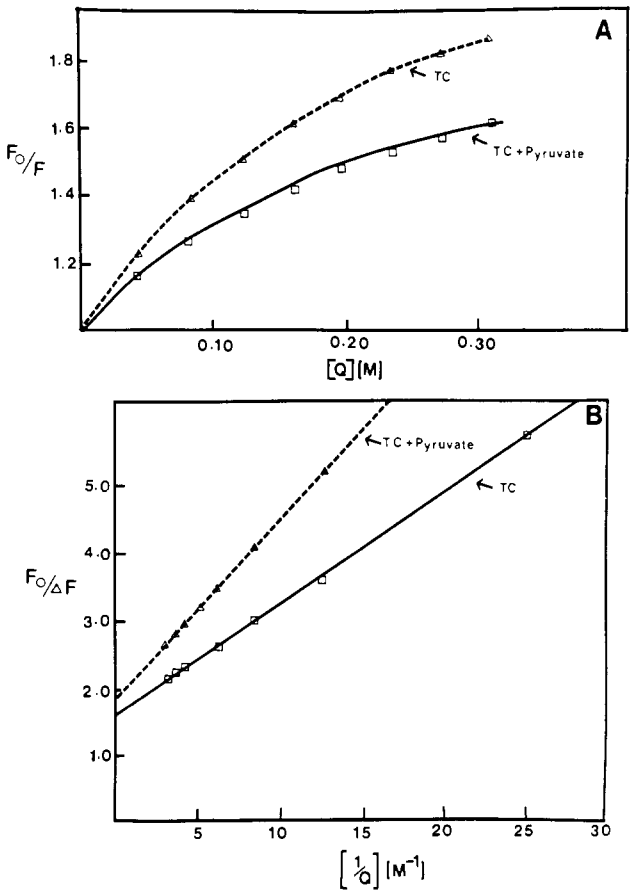

FIGURE 3: (A) Stern–Volmer plots of the fluorescence quenching with acrylamide (Q) of 0.083 μM transcarboxylase (△) and of the binary complex of 0.083 μM transcarboxylase and 9.9 mM pyruvate (□). Solvent is 250 mM sodium acetate, pH 5.5. (B) Modified Stern–Volmer plots of quenching by acrylamide (Q) of transcarboxylase (□) and the binary transcarboxylase–pyruvate complex (△).

with a downward curvature is obtained. This apparent nonlinearity indicates heterogeneity in the fluorescence emission

of Trps of transcarboxylase and a predominantly collisional quenching mechanism (Lehrer, 1971). The presence of a static factor in acrylamide quenching normally causes upward curvature in the Stern–Volmer plot (Eftink & Ghiron, 1981). However, for TC, which contains 90 Trps (Kumar et al., 1988), significant environmental heterogeneity can be expected, and some residues are likely to be more readily quenched than others. This possibility is supported by the quenching parameters derived from a replot of the quenching data according to the modified Stern–Volmer equation (Lehrer, 1971) (Figure 3B, Table I). A maximum fractional accessible fluorescence of 0.64 is derived for acrylamide quenching of TC fluorescence with an apparent K_Q of 9.45 M^{-1}.

The results from the acrylamide quenching studies performed with the TC (0.083 μM) in the presence of pyruvate (9.9 mM) are shown in Figure 3 and Table I. With TC, in the presence of pyruvate the Stern–Volmer plot displayed a nonlinear quenching with increasing concentration of acrylamide, suggesting a heterogeneous Trp microenvironment. The effective quenching constant (K_Q) decreased significantly from 9.45 (for the intact TC) to 6.73 M^{-1} (for the TC–pyruvate complex). Simultaneously, the maximum fractional accessible fluorescence, f_a, is reduced by 12%, suggesting that certain Trps are shielded sterically by pyruvate against acrylamide quenching.

Assessment of the Microenvironment of Trps of the Outer 5S Subunit both in the Absence and in the Presence of Pyruvate. Acrylamide quenching studies were performed with the 5S subunit both in the presence and in the absence of pyruvate, and the results are shown in Figure 4 and Table I. With the 5S subunit, the Stern–Volmer plot (Figure 4A) exhibits a nonlinear pattern, suggesting a heterogeneous Trp microenvironment. Additionally, ~74% of the tryptophanyl fluorescence was accessible for quenching with a K_Q of 6.55 M^{-1} (Figure 4B, Table I). In the presence of pyruvate, only 54% of the fluorescence was quenched with the collisional quenching constant of 5.94 M^{-1} (Table I). On the basis of the reduced quenchable fluorescence of the outer 5S subunit in the presence of pyruvate, it is suggested that certain Trps may be involved at or near the pyruvate binding site located on the 5S subunit of transcarboxylase.

Both pyruvate interaction and acrylamide quenching studies have indicated that the characteristics of the pyruvate binding site in the intact TC and in the 5S subunit are in general similar. Accordingly, in the subsequent chemical labeling studies involving Trps, only the outer 5S subunit has been investigated.

Identification of the Trps Involved at or near the Pyruvate Binding Site of the Outer 5S Subunit. We have carried out specific labeling of the Trps of the 5S subunit with 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl) (Scoffone et al., 1968) to aid in identifying the amino acid sequences around the Trp at the pyruvate binding site. The DNPS-labeled 5S subunit was digested with trypsin, and the DNPS-labeled tryptic peptides were fractionated by reverse-phase HPLC. As the DNPS functional group absorbs at 350 nm, the peptide elution was monitored at 350 nm. A typical elution profile of DNPS peptides of the 5S subunit is presented in Figure 5A. The incorporation of DNPS appears to be specific; one major (peak C) and several minor (peaks A, B, E, and F) DNPS-labeled peptides were seen. The broad peak G represents the strongly hydrophobic DNPS-Cl reagent.

Modification of the Trps of the 5S subunit by DNPS-Cl was also carried out in the presence of pyruvate using procedures similar to the one described above. The DNPS incorporation

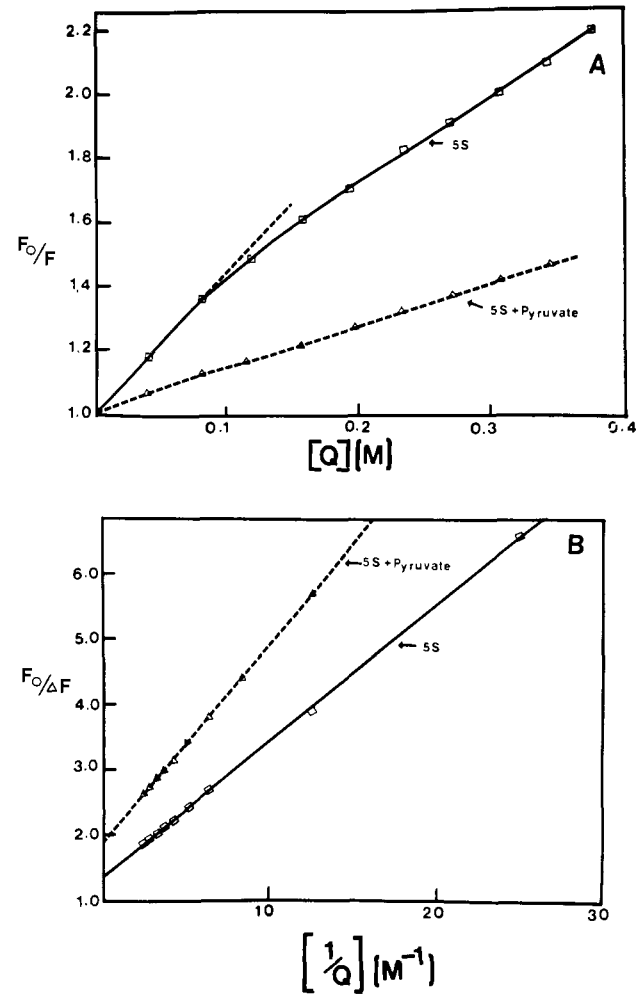

FIGURE 4: (A) Stern–Volmer plots of the fluorescence quenching with acrylamide (Q) of 0.6 μM outer 5S subunit (□) and 5S subunit (0.6 μM)–pyruvate (9.9 mM) complex (△). Solvent is 250 mM sodium acetate, pH 5.5. (B) Modified Stern–Volmer plots of the quenching with acrylamide (Q) of 5S subunit (0.6 μM) (□) and 5S subunit (0.6 μM)–pyruvate (9.9 mM) complex (△).

in this case was monitored by HPLC analysis of the tryptic peptides labeled with DNPS-Cl. As shown in Figure 5B, peptide C (of Figure 5A) was absent, suggesting that in the presence of pyruvate a specific Trp was inaccessible for labeling with DNPS-Cl. This evidence suggests that a Trp is at or close to the pyruvate binding site on the outer subunit of transcarboxylase.

Amino-terminal and amino acid analyses of the various DNPS-labeled peptides of Figure 5A have revealed that only peptide C was present in sufficient quantity for further sequence analysis. Peptide C was further purified on a C-18 reverse-phase column (Figure 6) and was found to be homogeneous as judged by a single Phe amino-terminal residue. The amino acid composition of the Phe peptide is shown in Table II. The sequence has been determined by gas-phase sequencing, and the complete sequence is

DISCUSSION

In this study we have probed the pyruvate binding region of transcarboxylase from *P. shermanii*, and the findings suggest that Trp-73 is involved at the pyruvate binding region of transcarboxylase. The binding of pyruvate to TC caused significant fluorescence quenching (Figure 1). Similar results

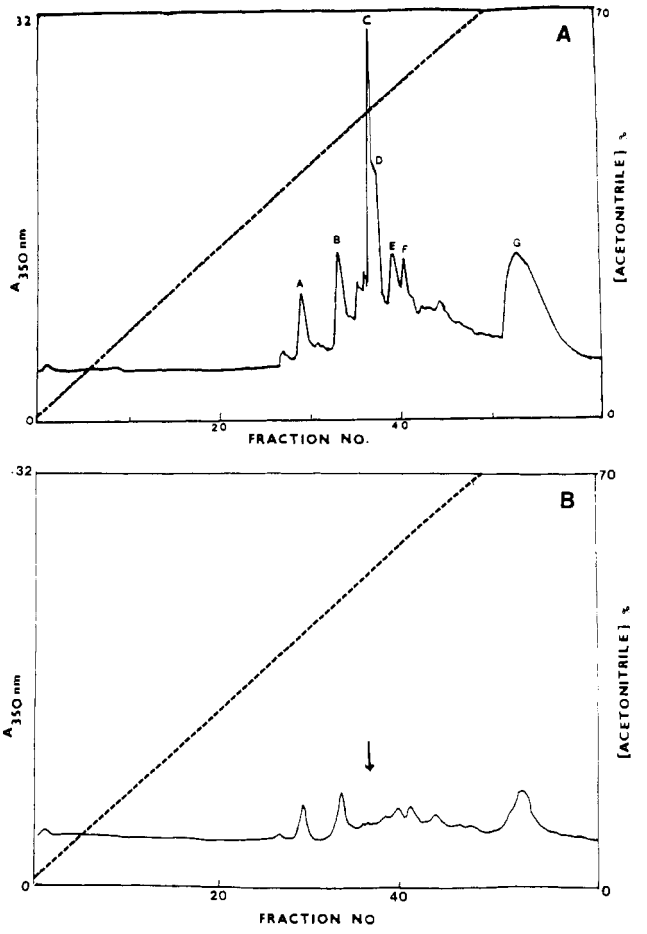

FIGURE 5: High-performance liquid chromatography profiles of the tryptic peptides of the DNPS-Cl-modified 5S subunit (A) and 5S subunit–pyruvate complex (B). A total digest was applied to a Synchropak C-4 reverse-phase column and eluted with a linear gradient of 0%–70% acetonitrile containing 0.1% TFA followed by isocratic elution with 70% acetonitrile containing 0.1% TFA. Absorption was monitored at 350 nm. The final yield of DNPS-labeled peptides except peptide C was about 6%, respectively.

Table II: Amino Acid Composition of DNPS-Labeled Peptide of the Outer 5S Subunit

amino acid	by analysis	by sequence
Asx	1.82 (2)	2
Glx	2.11 (2)	2
Arg	0.82 (1)	1
Pro	0.93 (1)	1
Leu	1.05 (1)	1
Phe	0.92 (1)	1
Trp[a]		(1)
total	8	9

[a] Trp was destroyed during acid hydrolysis. During sequencing, no residue was positively identified at the seventh cycle.

are also observed with the outer subunit (data not shown). The proportional decrease of the intrinsic fluorescence in response to pyruvate binding might be considered as a measure of its binding strength. This assumption has been applied to other protein–ligand interactions (Steiner, 1980; Omar & Schleich, 1981). In the present study, the apparent dissociation constants, 0.23 and 1.25 mM, were determined for the interaction of pyruvate with transcarboxylase. Similar apparent dissociation constants were obtained for the interaction of pyruvate with the outer 5S subunit (Table I). These observations suggest that the conformation of the pyruvate binding site located on the outer subunit is not influenced markedly by subunit–subunit interactions with the biotinyl 1.3S subunit and the central 12S subunit found in the intact enzyme. The

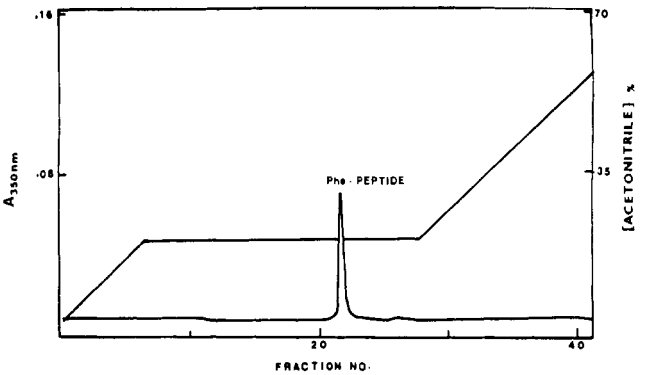

FIGURE 6: Purification of the major DNPS peptide (peptide C of Figure 5A) on a Synchropak RP-P C-18 column. The peptide was eluted isocratically with 25% acetonitrile containing 0.1% TFA. The elution was monitored at 350 nm for the DNPS peptide. The final recovery of peptide C was about 15%.

observation of a biphasic plot (Figure 2) giving rise to two apparent dissociation constants may suggest two binding sites for pyruvate with slightly different binding affinities. The preferential binding of pyruvate to one of the sites may bring about small conformational changes at the second binding site, thus generating apparent differences in the binding affinities for the two pyruvate binding sites of the dimeric outer 5S subunit.

Additional evidence for the involvement of Trp(s) in the pyruvate binding region has been provided by the acrylamide quenching studies of the enzyme carried out in the presence of pyruvate. The intact enzyme with a Trp content of 90 residues (Kumar et al., 1988) exhibited heterogeneous fluorescence emission with the maximum fractional accessible quenching, f_a, of 0.64 (Table I). A decrease ($\sim$12%) in f_a was observed with the whole enzyme in the presence of pyruvate, and with the outer subunit a $\sim$27% decrease in $f_{a(eff)}$ was observed. Pyruvate bound to either TC or the outer 5S subunit can reduce the accessibility of fluorophores to quenchers like acrylamide by steric blockage, by the induction of a conformational change (Hélène, 1977), or by the provision of an unfavorable electrostatic environment (Omar & Schleich, 1981). Even though interpretation of f_a values for multitryptophan proteins has several uncertainties (Eftink & Ghiron, 1981), we believe that it remains valid to conclude that a significant fraction of the Trp fluorescence of the enzyme (or of the outer subunit) as a result of pyruvate binding was not accessible for acrylamide quenching.

The use of Trp-specific DNPS-Cl for the modification of the outer 5S subunit has permitted the identification of a specific Trp that is sensitive to pyruvate binding. The incorporation of the DNPS group into the outer 5S subunit was found to be rather specific (about 4.7 mol of DNPS per mole of the monomer of the 5S subunit), and the tryptic peptides of the DNPS-labeled outer subunit were separated by HPLC. In general, we have consistently observed a poor recovery (ranging from 15 to 25%) of the DNPS-labeled peptides obviously due to strong interaction of these peptides with the reverse-phase columns used for separation. Significant incorporation of the DNPS label was found to be associated with a single Trp of peptide C (Figure 5A), and there was no such incorporation in the presence of pyruvate (Figure 5B). These observations have provided direct evidence for the involvement of Trp at or near the pyruvate binding region of the outer subunit.

The primary amino acid sequence of the outer subunit has been determined from the nucleotide sequence of its corresponding coding gene (Thornton et al., 1987; Samols et al.,

1988). Peptide C (Figure 5A) has been identified with residues 67–75 in the amino-terminal region, and the Trp modified by DNPS-Cl was identified as Trp-73. A comparison of the amino acid sequence around Trp-73 with the recently determined sequences from *Klebsiella* oxalacetate decarboxylase and yeast pyruvate carboxylase (Samols et al., 1988) shows that this region is highly conserved. For example, the N-terminal amino acids comprising residues 54–153 of the outer 5S subunit are 74% identical with the corresponding sequences of oxalacetate decarboxylase, which catalyzes a reaction with a very different metabolic function from those of either transcarboxylase or pyruvate carboxylase. The sequence of yeast pyruvate carboxylase is 50% identical when compared to the same region of the 5S subunit. All of these proteins interact with pyruvate, and thus it appears that region 67–75 may constitute part of the pyruvate binding pocket. It is possible that Trp-73 may be in close proximity to the Co^{2+} or Zn^{2+}, which has been suggested to participate in the catalysis (Northrop & Wood, 1969; Fung et al., 1974). It remains to be determined if selective modification of Trp-73 perturbs the NMR or EPR signal associated with the bound Co^{2+} of the outer subunit of transcarboxylase or affects the activity of the outer 5S subunit in the partial reaction.

Registry No. TC, 9029-86-1; Trp, 73-22-3; pyruvic acid, 127-17-3.

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