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Saccharomyces cerevisiae Phosphoenolpyruvate Carboxykinase: Physicochemical Characteristics of the Nucleotide Binding Site, As Deduced from Fluorescent Spectroscopy Measurements†

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ABSTRACT: *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] is inactivated by the fluorescent sulfhydryl reagent *N*-(iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS). The inactivation reaction follows pseudo-first-order kinetics with respect to active enzyme to less than 10% remaining enzyme activity, with a second-order inactivation rate constant of 2.6 min^{-1} mM^{-1} at pH 7.5 and 30 °C. A stoichiometry of 1.05 mol of reagent incorporated per mole of enzyme subunit was found for the completely inactivated enzyme. Almost complete protection of the enzyme activity and of dansyl label incorporation are afforded by MnADP or MnATP, thus suggesting that 1,5-IAEDANS interacts with an enzyme sulfhydryl group at the nucleotide binding site. The fluorescence decay of the AEDANS attached to the protein shows a single-exponential behavior with a lifetime of 18 ns. A comparison of the fluorescence band position and the fluorescence decay with those of the adduct AEDANS-acetylcysteine indicates a reduced polarity for the microenvironment of the substrate binding site. The quenching of the AEDANS moiety in the protein can be described in terms of a collisional and a static component. The rate constant for the collisional component is much lower than that obtained for the adduct in a medium of reduced polarity. These last results indicate that the AEDANS moiety is considerably shielded from the solvent when it is covalently attached to PEPCK.

Yeast phosphoenolpyruvate carboxykinase (PEPCK)[1] [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] catalyzes the reaction

$$ATP + OAA \xrightleftharpoons{Mn^{2+}} PEP + ADP + CO_2 \quad (1)$$

which is an important regulatory step in the biosynthesis of glucose from C_3 and C_4 precursors (Utter & Kolenbrander, 1972; Gancedo & Schwerzman, 1976). Most animal PEPCKs are GTP-dependent enzymes composed of single polypeptide chains of about 70 kDa, whereas the yeast enzyme is an ATP-dependent tetramer with a subunit molecular mass of 61.4 kDa (Utter & Kolenbrander, 1972; Stucka et al., 1988). The presence of quaternary structure and a clear specificity for adenine nucleotides have also been reported for the carboxykinases isolated from C_4 plants (Burnell, 1986) and from *Trypanosoma cruzi* (Urbina, 1987).

It has been communicated that sulfhydryl-directed reagents inactivate PEPCKs from various sources (Utter & Kolenbrander, 1972), and the presence of essential sulfhydryl groups is well documented in the sheep kidney (Barns & Keech, 1972) and rat liver enzymes (Carlson et al., 1978; Lewis et al., 1989).

The complete amino acid sequences of the enzymes from rat liver, chicken kidney, *Drosophila melanogaster* heads, and

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[1] Abbreviations: 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; AEDANS, *N*-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; PEP, phosphoenolpyruvate; OAA, oxalacetate; PEPCK, phosphoenolpyruvate carboxykinase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; P_i, inorganic orthophosphate.

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Saccharomyces cerevisiae have been informed (Beale et al., 1985; Cook et al., 1986; Gundelfinger et al., 1987; Stucka et al., 1988), and specific sequences for the nucleotide and PEP binding sites have been suggested for the cytosolic rat and chicken enzymes (Cook et al., 1986). A comparison of the primary structures of these enzymes indicates that monomeric PEPCKs share about 62–64% amino acid sequence similarity (Gundelfinger et al., 1987) whereas no significant similarities between the yeast gene sequence and sequences for the corresponding enzymes from rat liver or chicken were found (Stucka et al., 1988). Silverstein et al. (1979), by studying the interaction of 1,5-IAEDANS with hog liver PEPCK, have reported a low-polarity environment for the substrate binding site of the enzyme, in essential agreement with the hydrophobic nature for the putative substrate binding sites suggested for the chicken kidney and rat liver enzymes (Cook et al., 1986).

As a part of a study directed to establish structural relationships on monomeric and polymeric PEPCKs, we decided to explore the possibility of introducing a fluorescent label into the substrate binding site of yeast PEPCK, with the aim of defining its topographical properties. Analogues of ATP, i.e., the 2,3-dialdehyde derivative of 1-N^6-etheno-ATP, are not suitable for these studies because they bind with low affinity to yeast PEPCK (Saavedra et al., 1988).

We report here the specific binding of AEDANS to the *S. cerevisiae* PEPCK nucleotide binding site, and the characteristics of such binding site as deduced from fluorescence spectral characteristics and quenching studies. The fluorescent label employed in this study is selective for sulfhydryls and has a relatively long fluorescent lifetime (Hudson & Weber, 1973) for performing quenching studies conveniently (Tao & Cho, 1979).

Materials and Methods

Materials. ADP, ATP, PEP, malate dehydrogenase, NADH, Sephacryl S-200, 1,5-IAEDANS, Hepes, 2-mercaptoethanol, and *N*-acetylcysteine were obtained from Sigma Chemical Co. Acrylamide and SDS were from Bio-Rad Labs. All other reagents were of the purest commercially available grade. Organic solvents were spectral degree.

Preparation of the Enzyme. S. cerevisiae PEPCK was purified from yeast strain S288C according to Tortora et al. (1985), except that a gel filtration chromatographic step in Sephacryl S-200 (a 1.6 × 95 cm column eluted with 0.1 mM EDTA in 100 mM potassium phosphate buffer, pH 7.2 at 4 °C) was included between the DEAE-Sephacel and the AMP–Sepharose columns. The enzyme obtained (specific activity 40–42 units/mg of protein) showed a single band on polyacrylamide gel electrophoresis in the presence of SDS. The carboxykinase concentration was determined spectrophotometrically at 280 nm using a subunit molecular weight of 61 400 (Stucka et al., 1988) and an extinction coefficient of $\epsilon_{280}^{1\%} = 12.3$ cm^{-1} (Tortora et al., 1985). The enzyme assay was carried out as described previously (Malebrân & Cardemil, 1987).

Synthesis of 1,5-AEDANS–Acetylcysteine. This compound was synthesized as described by Hudson and Weber (1973). The product obtained had a single fluorescent spot on silica gel thin-layer chromatography plates, with ethanol as eluent.

Enzyme Inactivation with 1,5-IAEDANS. A solution containing the enzyme in 50 mM Hepes buffer, pH 7.5, was preincubated for 3 min at 30 °C (with or without protecting ligands), and then a certain amount of 1,5-IAEDANS in the same buffer was added to start the reaction. At given intervals, 5–10-μL aliquots were removed and assayed immediately for residual PEPCK activity. The addition of the modifier, 100–200 times diluted to the assay medium, had no effect on the enzyme activity or on the activity of the auxiliary enzyme, malate dehydrogenase.

Isolation and Characterization of the Labeled Enzyme. 1,5-IAEDANS (final concentration about 0.28 mM) was added to solutions of PEPCK in 50 mM Hepes buffer, pH 7.5 at 30 °C, containing 0.04–0.17 μmol of PEPCK subunits (final volume from 1.2 to 4.2 mL, respectively). At preestablished times (up to 4.75 min), the reactions were quenched by chilling the mixtures to 0 °C on ice, adding 100 mM 2-mercaptoethanol, and dialyzing against 100–200 volumes of 50 mM potassium phosphate buffer (pH 7.2, 4 °C) overnight, with a change of buffer after the first 4 h. The remaining enzyme activity was determined before the dialysis. The labeled enzyme was concentrated to 2–3 mg of protein/mL in Schleicher & Schuell collodion bags for protein concentration (molecular weight cutoff of 25 000) under vacuo, and the enzyme was further dialyzed against the same buffer. The extent of reaction was determined by using a molar extinction coefficient of 6100 M^{-1} cm^{-1} at 337 nm and 908 M^{-1} cm^{-1} at 280 nm (Hudson & Weber, 1973). The latter value was needed to correct for the contribution of the dansyl probe to the observed absorbance at 280 nm, which was used to determine the concentration of the protein. As a slight precipitate appeared after freezing and thawing, the labeled enzyme was kept at 0–4 °C for up to 3–4 weeks. Any precipitated protein was removed by ultrafiltration, as described above.

Fluorescence Lifetime and Quenching Studies. Fluorescence lifetime measurements were made with a phase/modulation fluorometer, GREG 200, equipped with a Pockels cell and frequency synthesizers. The lifetimes were measured with respect to glycogen as a reference compound. Measurements were made at 20 °C. The excitation wavelength was selected through a monochromator (2.5-nm slit). The emission was observed through appropriate band-pass filters. The data were analyzed by nonlinear least squares to obtain the best fits (Gratton et al., 1984; Hazletted et al., 1989).

Steady-state fluorescence measurements were done at 20 °C on a Perkin-Elmer LS-5 spectrofluorometer opering with 2.5-nm bandwidths for excitation and emission slits.

The quenching studies were performed by adding aliquots of a stock quencher solution to a cell containing the protein. Acrylamide fresh solutions were employed in all experiments. The decrease of the fluorescence intensity by the adding of the quencher was analyzed according to the modified Stern–Volmer equation (Lehrer & Leavis, 1978; Eftink & Ghiron, 1976):

$$I^0_F/I_F = (1 + K_{SV}[Q])\exp(V[Q]) = \tau^0/\tau \qquad (2)$$

where I^0_F and I_F are the intensities in the absence and presence of quencher, [Q], respectively, and K_{SV} and V are the dynamic and static quenching constants, respectively. Fluorescence lifetime values in the absence (τ^0) and presence (τ) of quencher only reflect the dynamic quenching process.

The steady-state fluorescence anisotropies (r) were measured with a Greg 200 spectrofluorometer equipped with calcite prism polarizers, and the anisotropy was calculated from

$$r = \frac{I_v - I_h}{I_v + 2I_h} \qquad (3)$$

where I_v and I_h are the intensities of the emitted light measured for the vertical and the horizontal axis, respectively. The anisotropies were obtained by varying the temperature and in a glycerol 70% solution. The excitation wavelength was 380 nm, where the anisotropy reaches a plateau value (Hudson & Weber, 1973).

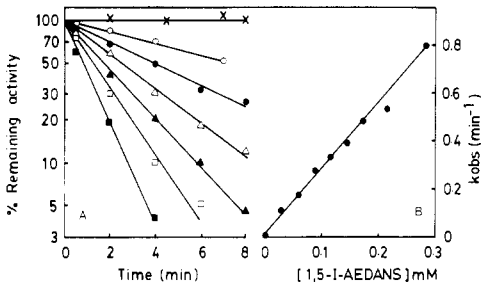

FIGURE 1: Inactivation of *Saccharomyces cerevisiae* PEPCK by 1,5-AEDANS. (A) The enzyme (1.7 μM subunits) was incubated at 30 °C in 50 mM Hepes buffer, pH 7.5, in the absence (×) or presence of 29 μM (○), 58 μM (●), 86 μM (△), 144 μM (▲), 216 μM (□), and 288 μM (■) 1,5-IAEDANS in a final volume of 0.2 mL. At the indicated times, aliquots of 5 μL were taken for the activity assays, as described under Materials and Methods. (B) Direct plot of the pseudo-first-order inactivation rate constant (k_{obs}) versus 1,5-IAEDANS concentration. The k_{obs} values were calculated from the data shown in (A), and from additional (not shown) data.

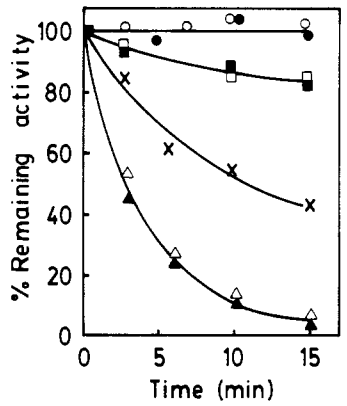

FIGURE 2: Effect of substrates on enzyme inactivation by 1,5-IAEDANS. The enzyme (1.4 μM subunits) was incubated under the conditions given in Figure 1 in the absence (○) or presence of 0.1 mM 1,5-IAEDANS (▲), 0.1 mM 1,5-IAEDANS plus 2 mM $MnCl_2$ (△), 0.1 mM 1,5-IAEDANS plus 2.5 mM PEP and 4 mM $MnCl_2$ (×), 0.1 mM 1,5-IAEDANS plus 1.25 mM ADP (□), 0.1 mM 1,5-IAEDANS plus 1.25 mM ATP and 2 mM $MnCl_2$ (■), or 0.1 mM 1,5-IAEDANS plus 1.25 mM ADP plus 2 mM $MnCl_2$ (●). At the indicated times, aliquots of 5–10 μL were taken for the activity assays, as described under Materials and Methods.

RESULTS

Inactivation of Yeast PEPCK by 1,5-IAEDANS. Incubation of the enzyme with 1,5-IAEDANS resulted in its progressive and total inactivation, which follows pseudo-first-order kinetics with respect to active enzyme, as shown in Figure 1A. The enzyme inactivation rate is a function of reagent concentration from 29 to 288 μM, and from the slope of the graph shown in Figure 1B, a second-order inactivation rate constant of 2.6 min^{-1} mM^{-1} could be calculated. Figure 2 shows that enzyme inactivation by 1,5-IAEDANS is effectively prevented by the combined presence of saturating concentrations of ADP (or ATP) plus $MnCl_2$ and, to a lesser degree, by PEP plus $MnCl_2$. These results suggest that the target amino acid residue of the sulfhydryl-directed reagent 1,5-IAEDANS may be located in (or close to) the nucleotide binding site of the enzyme. The observation that the protective effect of ADP is strengthened by the presence of $MnCl_2$ is in agreement with the conclusion that the kinetically active species in catalysis is the $MnADP^{2-}$ complex (Cannata & deFlombaum, 1974).

Determination of the Binding Stoichiometry. Yeast PEPCK was incubated with 1,5-IAEDANS as described under Materials and Methods, and the correlation between loss of enzyme activity and the number of moles of AEDANS bound per mole of enzyme subunit, assuming a subunit molecular weight of 61 400 (Stucka et al., 1988), is shown in Figure 3. It can be seen that there is a direct relation between loss of enzyme activity and incorporation of dansyl label, with a limiting value of 1.05 mol of AEDANS bound per mole of enzyme subunit at 0% residual enzyme activity. The nonspecific binding of AEDANS is very low, as it was found when an experiment similar to those described in Figure 3 was performed (except that the inactivation medium was also 2.0 mM each in ATP and $MnCl_2$), the incorporation of label after 4.75 min of reaction was only 0.05 mol/mol of enzyme subunit, while the enzyme retained 98% of its original activity. For the experiments described in Figure 3, the incorporation of AEDANS and the enzyme activity were 1.05 mol/mol of enzyme subunit and 1.6%, respectively, after 4.75 min of reaction.

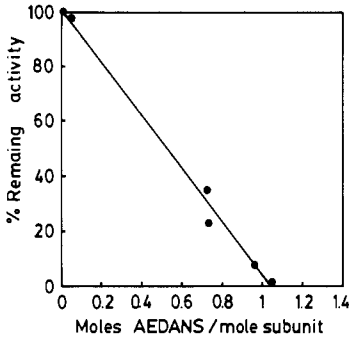

FIGURE 3: Relationship between enzyme activity and binding of AEDANS to *Saccharomyces cerevisiae* PEPCK. The enzyme samples (35–45 μM in subunits) were incubated at 30 °C in 50 mM Hepes buffer, pH 7.5, with 260–320 μM 1,5-IAEDANS. At definite times, the reactions were quenched and the remaining enzyme activity and the incorporation of dansyl label into the protein determined as described under Materials and Methods.

Emission Fluorescence Characteristics. The emission fluorescence spectra of AEDANS bound to the protein and to a small model compound, *N*-acetylcysteine, were measured in a series of solvents and ethanol/water mixtures. In the homogeneous solutions, the emission maxima fluorescence, as previously reported (Hudson & Weber, 1973), shifts continuously to the blue when the solvent polarity decreases. These shifts correlate fairly well with the empirical spectroscopic parameter $E_T(30)$ derived from the solvation band of pyridinium *N*-phenolbetaine (Reichard, 1979). On the other hand, the emission fluorescence spectra of AEDANS bound to the protein show maxima at 478 nm. This value interpolates to an $E_T(30)$ of 55 kcal/mol, corresponding to a mixture of ethanol/water (60% ethanol), a result that indicates a somewhat reduced polarity of the AEDANS moiety microenvironment.

The fluorescence lifetime of AEDANS–PEPCK measured by phase modulation fluorometry could be described by a two-component model with a lifetime of 18 ns and with a fractional intensity higher than 0.97 and a fixed component of 0.001 ns. This short component constitutes a correction for scattering light, not removed by the cutoff filters (Hazlett et al., 1989). Lifetime values around 18 ns have often been reported for AEDANS-labeled proteins (Tao & Cho, 1979; Tao & Lamkin, 1981; Chu et al., 1982; Cheung et al., 1983; Ajtai & Burghardt, 1987).

Fluorescence Quenching Experiments. In order to get further information on the solvent accessibility to the fluorescent probe, the quenching of the fluorescent probe by acrylamide was measured. These studies were carried out with AEDANS bound to the protein as well as with AEDANS bound to a small –SH-containing molecule. The quenching

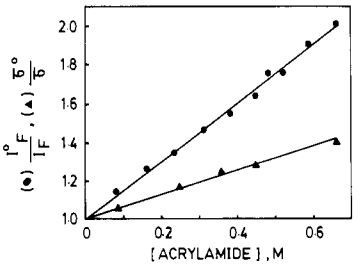

FIGURE 4: Stern–Volmer plots for the quenching of fluorescence of AEDANS-labeled PEPCK by acrylamide. (●) Steady-state fluorescence intensity; λ_{ex} = 340 nm, λ_{em} = 480 nm. (▲) Fluorescence lifetime. All the experiments were carried out in 50 mM phosphate buffer, pH 7.2, at 20 °C.

Table I: Fluorescence Quenching Rate Constants by Acrylamide and Fluorescence Lifetimes of the AEDANS Chromophore in Different Media[a]

sample	media	$k_q \times 10^8$ (M^{-1} s^{-1})	τ^b (ns)
AEDANS–PEPCK	50 mM KP_i, pH 7.2	0.35	18
AEDANS–PEPCK	40 mM Mn^{2+}/50 mM Hepes, pH 7.5	0.35	
AEDANS–acetylcysteine	50 mM KP_i, pH 7.2	10.8	10.5
	water	10.8	10.5
	ethanol/water (25)[c]	7.2	14.2
	ethanol/water (50)	4.9	18
	ethanol/water (70)	4.9	19
	ethanol/water (85)	5.4	24
	ethanol/water (100)	7.7	18.8
	N,*N*-dimethylformamide	3.5	11

[a] Excitation wavelength was 340 nm. k_q values were determined from the fluorescence lifetime, for the protein, and from the fluorescence intensity, for the model. [b] Determined under nitrogen atmosphere. [c] The number in parentheses indicates the percentage of ethanol (v/v) in the ethanol/water mixtures.

of AEDANS–PEPCK is shown in Figure 4 as Stern–Volmer plots. As can be seen, there is a difference between the intensity (I^0_F/I_F) and the lifetime (τ^0/τ) plots. This difference is an indication that both static quenching and dynamic quenching occur with acrylamide.

It is interesting to note that the fluorescence emission spectra were found to be unalterated by the presence of acrylamide up to 0.6 M and that the fluorescence lifetimes fit to a single component even at the higher acrylamide concentrations employed.

From the lifetime data, a dynamic quenching constant, $k_q\tau^0$ of 0.62 M^{-1} was obtained, and a quenching rate constant, k_q, of 3.5×10^7 M^{-1} s^{-1} is calculated. When this $k_q\tau^0$ value and the fluorescence intensity data are used, a static quenching constant of V = 0.62 M^{-1} is obtained.

The quenching of the AEDANS–acetylcysteine adduct was carried out using different solvents or solvent mixtures. In all cases, the Stern–Volmer plots were linear, and they showed a reasonable correlation between the intensity and the lifetime quenching data, proving that in homogeneous media the quenching of AEDANS by acrylamide is mostly a dynamic process. The values of the quenching rate constants obtained are included in Table I. These data show that k_q diminishes on going from water to a less polar medium. However, there is not a clear correlation with parameters that reflect the solvent polarity, as $E_T(30)$ (Reichard, 1979), the dielectric constant, the Kosower parameter (Kosower, 1958), or the π^* scale (Kamlet et al., 1977).

Fluorescence Steady-State Anisotropy. The steady-state emission anisotropies of protein-bound AEDANS determined

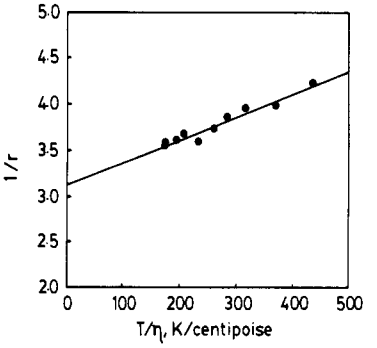

FIGURE 5: Fluorescence anisotropy of AEDANS-labeled enzyme. The data are presented as a Perrin plot, and they were obtained by varying the temperature. λ_{ex} = 380 nm.

at various temperatures are shown in Figure 5. The plot $1/r$ versus T/η was found to be linear over the temperature range 4–35 °C. The intercept of this plot gave a value of r_0 = 0.32. From the Perrin equation and a lifetime of 18 ns, a rotational correlation time of 67 ns at 20 °C is obtained. The overall correlation time, Φ, for a spherical hydrated macromolecule can be calculated by using eq 4. Assuming values of the

$$\Phi = \frac{M(v + h)\eta}{kT} \quad (4)$$

degree of hydratation, h, of 0.3 mL/g and the partial specific volume, v, of 0.73 mL/g, which are the values found for most proteins (Kuntz & Kauzman, 1974), the overall rotational correlation time for a spherical model of PEPCK (MW = 245 000) is 91 ns at 20 °C. This value is higher than that calculated from the anisotropy measurements shown in Figure 5.

The limiting anisotropy of AEDANS–PEPCK was also determined in 70% glycerol at 10 °C, obtaining a value of r_0 = 0.30, which approximates to that obtained from the temperature variation measurements.

Discussion

The data presented in this paper support the conclusion that the inactivation of *Saccharomyces cerevisiae* PEPCK by the fluorescent sulfhydryl-specific reagent 1,5-IAEDANS is due to modification of a functional amino acid residue located at the nucleotide binding site of the enzyme.

Low concentrations of this reagent completely inactivate the enzyme, and the inactivation reaction follows pseudo-first-order kinetics and is first order with respect to reagent, indicating that the inactivation proceeds in an all-or-none fashion. The protection afforded by ADP or ATP plus $MnCl_2$ of the inactivation caused by 1,5-IAEDANS indicates that the reagent is modifying a residue in or close to the nucleotide binding site of the enzyme. The observation that the protective effect of the substrates plus Mn^{2+} is greater than the effects of them alone is in agreement with the results of Cannata and de-Flombaum (1974), who have suggested that the true substrate for the enzymatic reaction is the cation–nucleotide complex. Further, the absence of protection by Mn^{2+} suggests that the Mn^{2+} binding site of the enzyme [for which there is evidence in *S. cerevisiae* PEPCK as well as in PEPCKs from several other sources (Malebrán and Cardemil (1987) and references cited thereof)] would not be located in the vicinity of the 1,5-IAEDANS interacting site. Analogously, Lewis et al. (1989) found the absence of divalent metal ion protection for cytosolic rat liver PEPCK inactivation by *N*-[7-(dimethylamino)-4-methyl-5-coumarinyl]maleimide. In this case, the –SH-directed maleimide derivative also interacts with the enzyme nucleotide binding site.

Our data on the binding stoichiometry of 1,5-IAEDANS to yeast PEPCK directly indicate that the loss of enzyme activity is correlated with the binding of one molecule of reagent per enzyme subunit, suggesting the specific modification of a single amino acid residue or the partial reaction of more than one residue, as discussed by Ray and Koshland (1961). Our data for the fluorescence decay, however, appear to indicate that there is no heterogeneity in the label microenvironment, which would be reflected by fluorescence components of different emission (Eftink & Ghiron, 1981). The possibility that the components may have very similar lifetimes cannot be ruled out. However, the homogeneous lifetime is present even at high acrylamide concentrations, a situation in which emission heterogeneity could be expected since the more exposed group would be preferentially quenched. All these facts, along with the known specifity of 1,5-IAEDANS for protein sulfhydryl groups (Hudson & Weber, 1973; Cheung et al., 1983), are compatible with the specific labeling of a -SH group per yeast PEPCK subunit, located in the enzyme nucleotide binding site. Titration experiments with DTNB carried out in both the native and SDS-denatured enzyme (E. Cardemil, unpublished results) indicate that, even when the total number of titrable -SH groups is close to 10 in both conditions, there is a small population of highly reactive -SH groups in the native enzyme. The presence of highly reactive functional -SH groups has been described in several monomeric, GTP-dependent, PEPCKs (Barns & Keech, 1972; Carlson et al., 1978), and recently Lewis et al. (1989) have identified Cys-288 as the hypereactive sulfhydryl group of the rat liver enzyme. It is possible that highly reactive -SH groups may be of critical importance either for the catalytic mechanism or for the maintenance of the active conformation of the protein, irrespective of the nucleotide specificity or the quaternary structure of the enzyme.

The remarkable shifts of the fluorescence spectra of the AEDANS moiety with media polarity can be used to obtain information on the micropolarity around the labeling site of the probe, and hence the polarity of the microenvironments of the substrate binding site. For this purpose, we have used the $E_T(30)$ parameter, since a good correlation was found for $E_T(30)$ with the spectral shifts for solvents of different characteristics (i.e., polar aprotic solvents as acetonitrile, protic solvents, and solvent mixtures). Our finding that the microenvironment sensed by the AEDANS chromophore attached to the PEPCK is similar to that of a mixture of ethanol/water (60% ethanol), based upon the $E_T(30)$ scale, indicates that the particular microenvironment for the protein in that region is of reduced polarity. These results are very similar to those reported by Silverstein et al. (1979), who observed a λ_{max} of 474 nm for AEDANS bound to the substrate binding site of hog liver PEPCK.

The low polarity detected by the emission characteristics of a protein-bound probe, however, does not necessarily imply that the probe is attached to a region at which the solvent access is restricted, since it is only the microenvironment polarity which is sensed by the fluorophore. Fluorescence quenching experiments of fluorescent probes attached to proteins have been used to determine the solvent accessibility to the probe, and acrylamide is one of the most employed quenchers (Eftink & Ghiron, 1981; Dieterich et al., 1979). Eftink et al. (1987) have reported that the quenching of 1,5-IAEDANS by acrylamide takes place through a charge transfer mechanism. In general, quenching rate constants of processes that occur through a charge transfer complex are dependent on the solvent polarity properties (Encinas & Lissi, 1985). Data given in Table I show that the fluorescence quenching of AEDANS–acetylcysteine by acrylamide decreases when the solvent is changed from water to less polar solvents or solvent mixtures. For this reason, this fact should be considered in the interpretation of the k_q values of a probe bound to a protein. The reduced value of k_q found for AEDANS–PEPCK could be an effect of the local microenvironments around the probe or an effect of the inaccessibility of the acrylamide molecule to the probe. As the value of k_q for AEDANS attached to PEPCK is even lower than that obtained for AEDANS–acetylcysteine in *N*,*N*-dimethylformamide (see Table I), the low value found for the protein cannot be only a consequence of the reduced micropolarity where the collision encounter AEDANS–acrylamide takes place. Hence, inaccessibility of acrylamide to the probe must play an important contribution. Low values of k_q are characteristics for the acrylamide quenching of tryptophan residues and for extrinsic fluorescent probes that are shielded from the medium solvent (Anderson & Hammes, 1983; Kuntenbach & Verjovski-Almeida, 1985; Eftink & Ghiron, 1976; McCublin & Key, 1980). The static component found for the acrylamide quenching of AEDANS–PEPCK, which is absent in the model compound, can be explained in terms of a partition of the quencher between the protein and the medium. Our results, then, appear to indicate that the quenching process takes place in a relatively apolar environment and the quencher accessibility to the probe is reduced.

The anisotropy steady-state data obtained in this work show that the correlation time determined for AEDANS attached to the protein is lower than that calculated for a hydrated sphere of the same molecular weight. This difference may be interpreted as the consequence of the rotational freedom of the probe around the bond linking it to the protein (Eftink, 1983; Sassaroli et al., 1982; Lakowitz, 1983).

Metal binding to proteins often induces conformational changes (Cheung, 1977; Kuntenbach & Verjovski-Almeida, 1985; Wu et al., 1989; Milne & Cook, 1979), and this possibility was explored in yeast PEPCK, where the binding of Mn^{2+} has been suggested (Malebrân & Cardemil, 1987). However, the fluorescence spectral parameters (λ_{max}, $\Delta\nu_{1/2}$), the fluorescence lifetime, and the acrylamide quenching were the same in the free protein or in the presence of Mn^{2+}. These findings show that there are no local changes in the probe neighborhood upon Mn^{2+} binding, thus implying that Mn^{2+} does not induce conformational changes or that they do not extend to the entire nucleotide binding site. Nevertheless, by use of proton relaxation rate techniques, electronic effects have been evidenced across long distances at the catalytic site of mitochondrial chicken liver PEPCK (Lee & Nowak, 1984).

In conclusion, we have been able to specifically label the nucleotide binding site of *S. cerevisiae* PEPCK with AEDANS. From comparison of the fluorescent characteristics of the bound probe to those of a model compound, we suggest that the probe is bound in a low-polarity region not easily accessible to the solvent. Our studies also appear to indicate that the movement of the probe is not completely restricted within the protein, suggesting a certain flexibility for the nucleotide binding site of the enzyme.

The finding of similar low-polarity microenvironments detected for both the yeast PEPCK and the hog liver enzyme (Silverstein et al., 1979) nucleotide binding sites is particularly interesting. Cook et al. (1986), based on the amino acid sequences of putative nucleotide binding sites of cytosolic chicken PEPCK, have suggested a hydrophobic nature for such protein regions. Recently, Lewis et al. (1989), by studying

the interaction of different maleimide derivatives with cytosolic rat liver PEPCK, have suggested a hydrophobic microenvironment for the enzyme nucleotide binding site. Thus, it is quite possible that monomeric and tetrameric PEPCKs—even when it has been informed that they do not share significant similarities in the protein primary structures (Stucka et al., 1988)—may have common features in their secondary or tertiary structures. More studies are needed in order to test this possibility.

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Registry No. PEPCK, 9073-94-3; 1,5-IAEDANS, 36930-63-9; MnADP, 69828-68-8; MnATP, 56842-80-9; 1,5-AEDANS-acetylcysteine, 50402-65-8.

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