Saccharomyces cerevisiae Phosphoenolpyruvate Carboxykinase: Revised Amino Acid Sequence, Site-Directed Mutagenesis, and Microenvironment Characteristics of Cysteines 365 and 458[†,▽]

Hans Krautwurst,[‡] María Victoria Encinas,[‡] Frank Marcus,[§] Steven P. Latshaw,[∥] Robert G. Kemp,[∥] Perry A. Frey,[⊥] and Emilio Cardemil*[,‡]

Departamento de Ciencias Químicas, Facultad de Química y Biología, Universidad de Santiago de Chile, Casilla 307-2, Santiago, Chile, Chiron Corporation, Emeryville, California 94608-2916, Department of Biological Chemistry, University of Health Sciences, The Chicago Medical School, North Chicago, Illinois 60064-3095, and Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53705-4098.

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ABSTRACT: Two cysteine residues in phosphoenolpyruvate (PEP) carboxykinase from *Saccharomyces cerevisiae* [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] the modification of which leads to enzyme inactivation have been subjected to site-directed mutagenesis. PEP carboxykinase is inactivated by alkylation of Cys^{365} or Cys^{458}; however, mutation of either or both of these residues to serine has little effect on the enzymatic activity. These results eliminate any possible catalytic function for these cysteinyl residues. In the course of this work, discrepancies in the published nucleotide sequence of the *S. cerevisiae* PEP carboxykinase gene were detected that alter the deduced amino acid sequence. Several of these descrepancies were verified through the sequencing of proteolytic peptides. Our results indicate that the protein corresponds to a 549 amino acid polypeptide and that the positions previously assigned to Cys^{364} and Cys^{457} correspond to Cys^{365} and Cys^{458}. The individual reactivities and the microenvironment characteristics around these sulfhydryl groups were investigated by their selective modification with the fluorescent reagent *N*-(1-pyrenyl)maleimide (PyM). Our findings indicate that Cys^{458} is 7-fold more reactive toward the sulfhydryl-directed probe than Cys^{365}, while quenching experiments of PyM-labeled mutant enzymes suggest that the former residue is located in a region more accessible to water than the latter.

The conversion of oxaloacetate into phosphoenolpyruvate (PEP)[1] in *Saccharomyces cerevisiae* is catalyzed by an ATP-dependent PEP carboxykinase [ATP:oxaloacetate carboxy-lyase [(transphosphorylating), EC 4.1.1.49] and is one of the first steps in the biosynthesis of glucose from C_3 and C_4 precursors (Utter & Kolenbrander, 1972; Muller et al., 1981).

On the basis of chemical modification experiments, it had been inferred that this enzyme contains reactive cysteinyl residues in the substrate binding region. In previous research we have shown that the fluorescent reagents 1,5-I-AEDANS and IANBD specifically react with $Cys^{365(2)}$ (Alvear et al., 1992, 1994), while pyrenyliodoacetamide labels Cys^{365} and $Cys^{458(2)}$ (Rojas et al., 1993). In the latter case, close proximity of the reactive residues has been deduced from the observation of pyrene excimer emission in the protein labeled with 2 mol of probe per mole of enzyme subunit. Our studies indicate that the microenvironment around the dansyl probe attached to Cys^{365} has a rather low polarity, and the protein matrix shows some flexibility in this region (Encinas et al., 1990). The different reactivities of Cys^{365} and Cys^{458} against alkylating reagents point to different characteristics of the protein matrix in that region; however, the lack of an appropriate probe to selectively label Cys^{458} has precluded the obtainment of comparable information for its microenvironment.

Research on PEP carboxykinases from several other organisms also indicates the presence of reactive sulfhydryl groups (Barns & Keech, 1972; Silverstein et al., 1979; Makinen & Nowak, 1989; Chen et al., 1991). For the cytosolic rat liver enzyme Lewis et al. (1989) have identified Cys^{288} as the most reactive thiol against *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide. Later, Lewis et al. (1993) and Rojas et al. (1993) identified additional cysteinyl residues, also probably located within the active site region.

In the present work, we have prepared mutant *S. cerevisiae* PEP carboxykinases containing the replacements C365S, C458S, and C365S/C458S with the double purpose of analyzing the possible catalytic role of these residues and selectively introducing appropriate fluorescent probes linked to each individual cysteine residue to obtain physical information about this protein region. Part of this work has been previously described (Cardemil et al., 1994).

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[‡] Universidad de Santiago de Chile.

[§] Chiron Corporation.

[∥] The Chicago Medical School.

[⊥] University of Wisconsin.

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1- naphthyl)ethylenediamine; IANBD, 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole; PEP, phosphoenolpyruvate; PyIA, *N*-(1-pyrenyl)iodoacetamide; PyM, *N*-(1-pyrenyl)maleimide; PyM-Ac, *N*-(1-pyrenyl)-maleimide-*N*-acetyl-L-cysteine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

[2] Formerly Cys^{364} and Cys^{457}.

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MATERIALS AND METHODS

Materials. Restriction enzymes, DNA ligase, DNA kinase, T4 DNA ligase, and T7 DNA polymerase were purchased from Promega Corp. or Boehringer Mannheim. The Sequenase version 2.0 DNA sequencing kit from U.S. Biochemical Corp. was used for sequencing. [α-^{35}S]dATP (1500 Ci/mmol) was obtained from Amersham Corp. Sequencing grade trypsin and endoproteinase Glu-C were from Boehringer Mannheim. PyM was from Molecular Probes. All other reagents were of analytical or molecular biological grade or of the purest commercially available grade. Organic solvents were spectral grade. The *N*-(1-pyrenyl)maleimide adduct with *N*-acetyl-L-cysteine was synthesized as described by Wu et al. (1976). Plasmid pMV7, consisting of a full-length cDNA clone of the *S. cerevisiae* PEP carboxykinase gene, covering the entire coding region, cloned into the *Hin*dIII/*Eco*RI sites of YEp352 (Valdés-Hevia et al., 1989) was employed. The insert contained 334 nucleotides in the 5′-untranslated region, the entire coding region, and 357 nucleotides in the 3′-untranslated region.

Construction of the Mutant Clones. Oligonucleotides and sequencing primers were synthesized by an automated DNA synthesizer (Applied Biosystems, Model 380A). After digestion with *Hin*dIII/*Eco*RI, the PEP carboxykinase gene was subcloned into M13mp19 by standard techniques (Sambrook et al., 1989). Uracil-containing ssDNA was prepared by using *Escherichia coli* host strain CJ236 (*ung*$^-$) to culture the M13mp19 PEP carboxykinase clone (Kunkel, 1985). The ssDNA was used as the template and hybridized to the phosphorylated oligonucleotides that contained the mismatched nucleotides that coded for either Ser^{365} or Ser^{458}. Mutagenesis was carried out according to Kunkel et al. (1987). After hybridization, the second strand was synthesized using T7 DNA polymerase and T4 DNA ligase. The dsDNA was transformed into *E. coli* JM109. The double-mutant DNA was obtained by site-directed mutagenesis of the previously mutated M13mp19-cloned PEP carboxykinase gene containing the appropriate base replacements that code for Ser^{365}. The plaques of mutant cDNA were identified by sequencing the appropriate regions; to confirm the desired mutation, the coding sequence between sites *Sal*I and *Eco*RI was sequenced (Sanger et al., 1977). After digestion with *Sal*I/*Eco*RI, the mutant cDNAs were subcloned into a similarly digested pMV7 and transformed into the PEP carboxykinase-deficient yeast strain PUK-3B (*MAT* α pck ura3) by the lithium acetate method (Ito et al., 1983), as modified by Ausabel et al. (1987).

The transformed yeast was grown under selective conditions on agar plates lacking uracil to prevent plasmid loss and using glucose as carbon source. Isolated colonies of all three mutants were able to grow in liquid cultures using pyruvate as carbon source, thus indicating the presence of active PEP carboxykinase. Precultures thus obtained were transferred to 1 L of rich medium containing 2% ethanol and grown for 48 h at 30 °C, and the enzymes were purified using the same procedure employed for the wild-type enzyme (Cardemil et al., 1990). All enzymes showed a single band on SDS−PAGE and had specific activities in the range 40−50 units/mg of protein. PEP carboxykinase concentration was determined spectrophotometrically at 280 nm using the extinction coefficient $\epsilon^{1\%} = 12.3$ cm^{-1} (Tortora et al., 1985).

Assay and Kinetic Studies. The standard assay described by Malebrán and Cardemil (1987) was used. One unit of enzyme activity is defined as the amount which produces 1 μmol of oxaloacetate/min. To obtain the kinetic parameters, enzyme activity was measured as the concentration of either substrate was varied. Maximal velocity and the apparent K_m were determined by fitting the data to the Michaelis−Menten equation with the EZ-FIT program of Perrella (1988).

Reaction with N-(1-Pyrenyl)maleimide and Peptide Sequencing. The modification reactions were carried out in 50 mM Hepes buffer, pH 7.5. A freshly prepared solution of PyM in dimethylformamide was used, and the concentration was determined spectrophotometrically (Wu et al., 1976). The added aliquots never exceeded 4% of the final volume. Because of the high reactivity of the sulfhydryl groups, the kinetics of enzyme inactivation by PyM was followed at 0 °C and not at 20 °C, using similar concentrations of enzyme and pyrene derivative. For labeling experiments, solutions of PEP carboxykinase (16−20 μM in subunits) were reacted at 20 °C with a 1.2-fold molar excess of PyM in a total volume of 1 mL. After 1−3 min, the reactions were quenched by chilling on ice and adding 100 mM 2-mercaptoethanol, and the reaction mixtures were then dialyzed against 50 mM Hepes buffer, pH 7.5, for 16 h at 4 °C in the dark. The extent of labeling was determined by using molar extinction coefficients of 4×10^4 M^{-1} cm^{-1} at 345 nm and 2.5×10^4 M^{-1} cm^{-1} at 280 nm for the pyrenyl chromophore (Wu et al., 1976). The labeled carboxykinases were carboxymethylated (Mayes, 1984), dialyzed against 50 mM NH_4HCO_3 at pH 8.5, and then subjected to proteolysis with 1% trypsin for 14 h at 30 °C. The peptides were analyzed by HPLC using a C_{18} LiChrocart reverse-phase column with acetonitrile gradients. The elution was monitored by absorbance and by fluorescence emission, as described in the corresponding figure captions. Selected fractions were collected and concentrated under a N_2 stream at 30 °C, and each was rechromatographed on a reverse-phase Supelco C_4 column, which was eluted under the same conditions. The purified fractions were subjected to amino acid sequence analysis as previously described (Rojas et al., 1993; Alvear et al., 1994).

Fluorescence Studies. Fluorescence lifetime measurements were carried out at 20 °C by multiple-frequency phase fluorometry, using a GREG 200 multifrequency and phase-modulation fluorometer. The samples were excited with the pulse of a Liconix 4042NB He−Cd laser (325 nm). The emission was observed through a cutoff filter of 400 nm. Phase angles and modulation ratios were measured at several frequencies, and the data were analyzed for heterogeneity using analysis routines supplied with the instrument (ISS Inc.) (Jameson et al., 1984). A scattering component, 0.001 ns, was routinely fixed in the analysis (Hazlett et al., 1993) and represented no more than 5% of the signal. In order to avoid possible effects of Brownian rotation on the measured lifetimes, magic angle polarization was used for all measurements. Me_2POPOP in ethanol ($\tau = 1.45$ ns) was used as reference lifetime standard. Steady-state fluorescence measurements were done at 20 °C on a Perkin-Elmer LS-5 spectrofluorometer with 2.5-nm excitation and emission slits. Quenching experiments were performed by adding aliquots of concentrated freshly prepared quencher solutions. The decrease of the fluorescence intensity or the lifetime was measured as a function of the quencher concentration.

RESULTS AND DISCUSSION

Site-Directed Mutagenesis, Sequencing of the M13-Cloned S. cerevisiae PEP Carboxykinase Gene, and Revised Amino Acid Sequence of the Protein. Mutagenesis was carried out using the M13-cloned PEP carboxykinase gene as described under Materials and Methods. Each mutation was confirmed by complete sequencing the *Eco*RI−*Sal*I restriction fragment containing the mutation.

In the course of sequencing these fragments, discrepancies with the published *S. cerevisiae* PEP carboxykinase gene (Stucka et al., 1988) appeared. These findings prompted us to completely sequence the M13-cloned gene, as well as portions of the noncoding sections included in the *Hin*dIII−*Eco*RI fragment, ranging from nucleotide position 200 up to 2227 of Stucka et al. (1988). The discrepancies found were as follows: additional C after position 618; additional GA after position 653; GC instead of CG in positions 699 and 700; C instead of A in position 1298; no A after position 1962; C instead of A in positions 1996, 2058, and 2145; additional T after position 2184; lack of A after position 2216; lack of AAAA after position 2220. All discrepancies in the coding region (336−1994) were confirmed by sequencing the (−) strand of the gene cloned in pMV7.

The corrections found in the coding region mandate alterations in the predicted amino acid sequence of the protein, so that the corrected sequence is that shown in Figure 1. To further verify some of these changes, several proteolytic peptides obtained from the native protein were sequenced, as also indicated in Figure 1. For all sequenced peptides, agreement was found between the newly predicted sequence and the actual amino acid sequence. The only regions where discrepancies between our results and the data of Stucka et al. (1988) could not be verified through peptide sequencing are the protein carboxyl end and His^{322}. Considering these results, the protein comprises 549 amino acids, with a calculated molecular mass of 60 983 Da. Consequently, the reactive cysteine residues previously identified (Alvear et al., 1992; Rojas et al., 1993) correspond to Cys^{365} and Cys^{458} and will be referred to as such in this paper. All changes lie in regions with little sequence similarity to other PEP carboxykinases (Østerås et al., 1991). Recently, Gaillon and Dujon (1994) have communicated the nucleotide sequence of *S. cerevisiae* chromosome XI reading frame ORFYKR097 (2641 bases), of which 1647 bases correspond to the PEP carboxykinase gene. The predicted amino acid sequence for the protein is in complete agreement with our results.

Expression, Purification, and Characterization of Mutant PEP Carboxykinases. To achieve expression of the mutated PEP carboxykinase genes, the respective mutagenized *Eco*RI-*Sal*I fragments were subcloned into pMV7, which was subsequently transformed into PEP carboxykinase-deficient yeast strain PUK-3B. The recombinant PUK-3B cells were grown using ethanol as carbon source, and the enzymes were purified by a previously described procedure (Cardemil et al., 1990). All proteins showed a single band in SDS−PAGE with the same migration as the native PEP carboxykinase subunit (not shown). The Cys→Ser replacements were confirmed through protein sequencing of PyM-labeled peptides obtained from the mutated enzymes (see below).

The apparent K_m and V_{max} were determined for all enzymes and are presented in Table 1, which shows that neither the single nor the double mutant enzymes present changes in these parameters. Consequently, the present investigation shows that the inactivation of *S. cerevisiae* PEP carboxykinase by chemical modification of Cys^{365} or Cys^{458} with sulfhydryl reagents is not due to the loss of chemical reactivity of these residues. While the mutation of Cys^{365} and Cys^{458} to Ser^{365} and Ser^{458} slightly reduced the volume of the amino acid side chain from a sulfhydryl group to a less bulky hydroxyl group, chemical modification introduces a much bulkier group on the sulfur of the reactive cysteines and increases the volume. Considering these arguments and the fact that wild-type enzyme is not inactivated or labeled

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  1 MSPSKMNATVGSTSEVEQKIRQELALSDEVTTIRRNAPAA  40

 41 VLYEDGLKENKTVISSSGALIAYSGVKTGRSPKDKRIVEE  80

                    **********
 81 PTSKDEIWWGPVNKPCSERTWSINRERAADYLRTRDHIYI 120

                  *
121 VDAFAGWDPKYRIKVRVVCARAYHALFMTNMLIRPTEEEL 160

161 AHFGEPDFTVWNAGQFPANLHTQDMSSKSTIEINFKAMEM 200

201 IILGTEYAGEMKKGIFTVMFYLMPVHHNVLTLHSSANQGI 240

241 QNGDVTLFFGLSGTGKTTLSADPHRLLIGDDEHCWSDHGV 280

281 FNIEGGCYAKCINLSAEKEPEIFDAIKFGSVLENVIYDEK 320

                  *
321 SHVVDYDDSSITENTRCAYPIDYIPSAKIPCLADSHPKNI 360

361 ILLTCDASGVLPPVSKLTPEQVMYHFISGYTSKMAGTEQG 400

401 VTEPEPTFSSCFGQPFLALHPIRYATMLATKMSQHKANAY 440

441 LINTGWTGSSYVSGGKRCPLKYTRAILDSIHDGSLANETY 480

481 ETLPIFNLQVPTKVNGVPAELLNPAKNWSQGESKYRGAVT 520

                        ******
521 NLANLFVQNFKIYQDRATPDVLAAGPQFE            549
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FIGURE 1: Revised amino acid sequence for *S. cerevisiae* PEP carboxykinase. The amino acid sequence was deduced from the nucleotide sequence of the enzyme gene cloned in pMV7 and is essentially that previously published by Stucka et al. (1988), except for changes denoted with asterisks (see text for additional details). Shown underlined are regions confirmed by direct amino acid sequencing of isolated peptides, obtained after trypsin or endoproteinase Glu-C treatment, as described in Materials and Methods.

Table 1: Kinetic Parameters for Wild-Type and Cys→Ser Mutant *S. cerevisiae* PEP Carboxykinases[a]

	apparent K_m			
enzyme	ADP (μM)	PEP (μM)	CO_2 (mM)	V_{max} (units/mg)
native	67 ± 10	244 ± 51	17 ± 2	72 ± 6
C365S	79 ± 11	211 ± 59	16 ± 2	78 ± 6
C458S	84 ± 10	214 ± 38	16 ± 3	69 ± 5
C365S/C458S	95 ± 9	262 ± 26	21 ± 3	74 ± 6

[a] Kinetic constants were determined as indicated in Materials and Methods. Values given are the mean ± SE of three separate experiments.

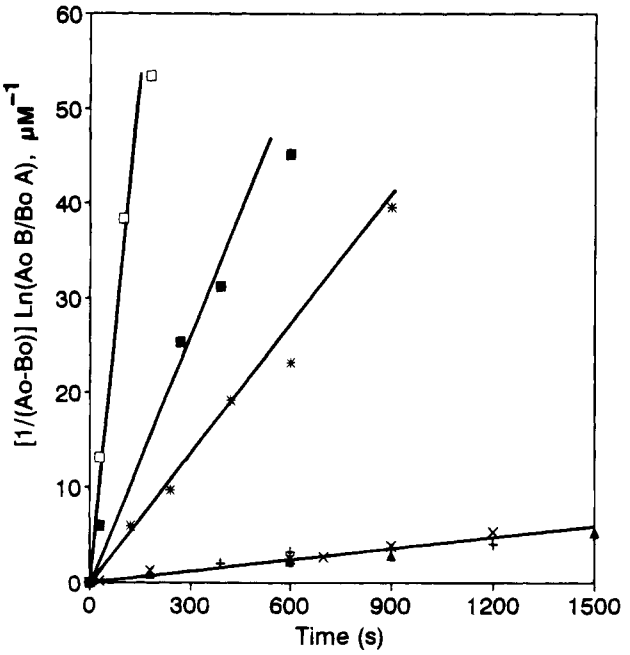

FIGURE 2: Second-order inactivation kinetics of wild-type and mutant PEP carboxykinases by PyM and the protection afforded by MnADP. The enzymes (6−10 μM in subunits) in 50 mM Hepes buffer, pH 7.5 at 0 °C, were incubated with a 1.2-fold molar excess of PyM in a total volume of 0.12 mL, and at appropriate times 5−10-μL aliquots were withdrawn and assayed for residual enzyme activity. Controls without PyM showed negligible inactivation. The data show the inactivation of wild-type, C365S, and C458S PEP carboxykinases in the absence (■, □, *, respectively) and the presence (+, ×, ▲, respectively) of 1.25 mM ADP plus 2.0 mM $MnCl_2$. Lines are least squares fit to the data.

by several sulfhydryl-directed reagents in the presence of MnADP (Alvear et al., 1992, 1994; Rojas et al., 1993)—it is reasonable to conclude that Cys^{365} and Cys^{458} are spatially near the nucleotide binding site. Chemical modification of these residues would inhibit the access or binding of substrates to the catalytic site. This point could be experimentaly tested by monitoring substrate binding to the modified enzymes. In the avian liver PEP carboxykinase, Makinen and Nowak (1989) have provided evidence through the labeling of the most reactive cysteinyl group of the enzyme with a spin-labeled iodoacetate that it is located about 10 Å from bound Mn^{2+}, thus indicating that this reactive cysteine is close to but not at the active site of the enzyme. Recent work on *E. coli* PEP carboxykinase (Bazaes et al., 1993) has shown that even when this enzyme has four cysteinyl residues, it is not inactivated by a number of sulfhydryl-directed chemical reagents, thus also pointing to a lack of catalytic relevance for sulfhydryls in PEP carboxykinases.

Inactivation and Labeling of Native and Mutant S. cerevisiae PEP Carboxykinases with PyM. Native, C365S mutant, and C458S mutant PEP carboxykinases were rapidly inactivated by the addition of stoichiometric concentrations of PyM. A simultaneous time-dependent increase in the fluorescence intensity of the pyrene monomer emission was detected in all cases. The time courses of inactivation followed clear second-order kinetics (Figure 2), with rate constants highly dependent on the reactive sulfhydryl group, as can be seen in Table 2. These results indicate that different sulfhydryl groups are reacting with PyM, C365S PEP carboxykinase being 7-fold more reactive than the

Table 2: Second-Order Inactivation Rate Constants for the Reaction of PyM with Native and Mutant *S. cerevisiae* PEP Carboxykinases[a]

enzyme	$k(\times 10^2 M^{-1} s^{-1})$	$k(\times 10^2 M^{-1} s^{-1})$[b]
native	7.6	0.34
C365S	31.0	0.42
C458S	4.3	0.35
C365S/C458S	0.4	nd

[a] Rate constants were determined in 50 mM Hepes buffer, pH 7.5, at 0 °C, except for the double mutant where the value at 30 °C is given. [b] Measured in the presence of 1.25 mM ADP plus 2.0 mM $MnCl_2$; nd, not determined. All conditions were as described in Materials and Methods.

C458S enzyme. As shown in Table 2, enzyme inactivation was effectively prevented by the combined presence of saturating concentrations of ADP plus $MnCl_2$, in agreement with previous results (Rojas et al., 1993; Alvear et al., 1994).

To determine the degree of labeling, enzyme samples were incubated for 1−3 min at 20 °C under the same experimental conditions with the fluorescent reagent, and the labeled proteins were then purified. Labeling stoichiometries (see Materials and Methods) were 0.95, 0.63, 0.66, and 0.44 mol of reagent incorporated/mol of enzyme subunit for the native, C365S, C458S, and double mutant PEP carboxykinases, respectively. Additional experiments (not shown) indicated similar stoichiometries after 10−12 min of reaction. On the basis of the different chemical reactivity of these sulfhydryl groups, it could be expected that different degrees of labeling would be found if labeling experiments were performed at lower temperatures. Similar experiments performed by incubating the native, C365S, and C458S PEP carboxykinases in the presence of saturating concentrations of ADP plus $MnCl_2$ showed low pyrene incorporation.

The PyM-labeled enzyme samples were subjected to tryptic digestion and HPLC analysis of the labeled peptides. The chromatogram of the native enzyme showed two main fluorescence peaks (Figure 3), while only one major peak appeared when peptides of the mutant enzymes were analyzed. The elution times for peaks 1 and 2 derived from the PyM-labeled native enzyme were identical with those originating from the C365S and C458S mutant enzymes, respectively. A similar analysis of the double mutant enzyme showed the complete absence of peaks 1 and 2 and the presence of two additional peaks at 93.6 and 104 min (not shown). These two peaks eluted between the positions of peaks 1 and 2, and completely separated from the elution positions of standards PyM-Ac and PyM-mercaptoethanol. It can also be seen in Figure 3 that peak 2 is clearly composed of two closely migrating components, while peak 1 appears as a single component. The same chromatogram was obtained when the pyrenyl-containing peptides were detected by monitoring their absorption at 344 nm. Peak 1 and the two components of peak 2 were collected and repurified in a C_4 reverse-phase HPLC column. This procedure led to the resolution of peak 1 into two closely migrating fluorescent components, which were individually collected. The samples were subjected to amino acid sequence determination. The analysis of components of peak 1 showed identical sequences corresponding to the peptide X-Pro-Leu-Lys. Peak 2 showed the sequence Asn-Ile-Ile-Leu-Leu-Thr-X-Asp-Ala-Ser-Gly-Val-Leu-Pro-Pro-Val-Ser-Lys. In these sequences, X stands for an unidentified component, which might have derived from the PyM-adduct. According to the protein

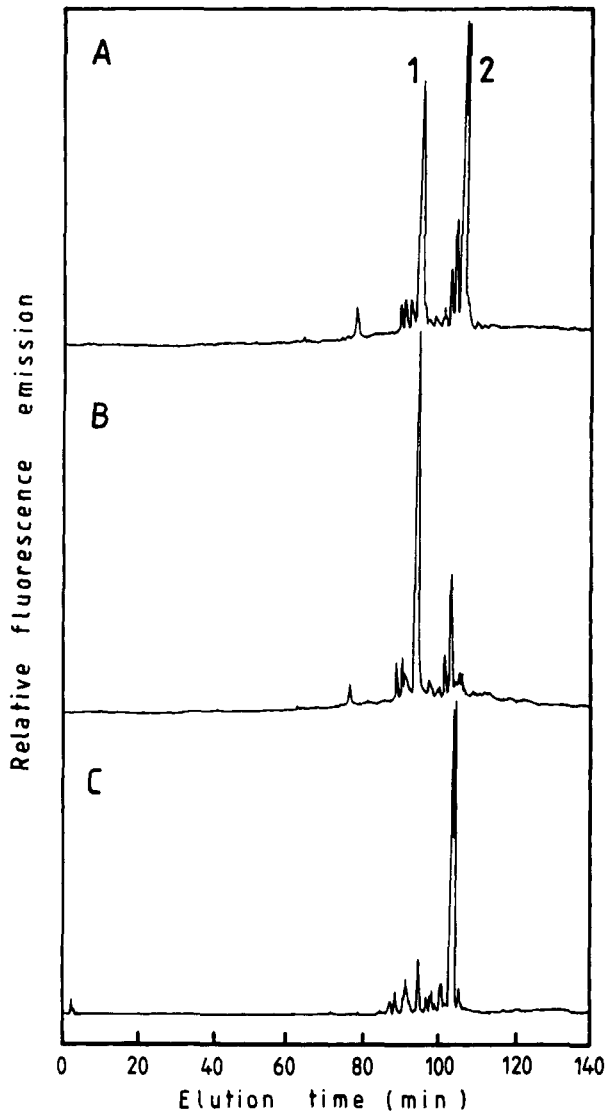

FIGURE 3: HPLC resolution of PyM-labeled tryptic peptides from wild-type and mutant PEP carboxykinases. (A) Native enzyme; (B) C365S enzyme; (C) C458S enzyme. Peptides were eluted with a linear gradient of 0−60% acetonitrile in 0.05% trifluoroacetic acid from 20 to 140 min, using a reverse-phase C_{18} column. The fluorescence at 400 nm was used to monitor the pyrene-containing peptides. The excitation was 340 nm.

sequence, these positions correspond to Cys^{458} and Cys^{365}, respectively. These results indicate that the mutagenesis work led to the expected Cys→Ser substitutions, and that PyM incorporation to Cys^{365} and Cys^{458} occurs in a mutually exclusive way.

In order to investigate the origin of the two components of the peaks labeled with PyM, we measured the fluorescence spectra of the labeled native PEP carboxykinase under different pH conditions. The spectrum of the native protein in Hepes buffer, pH 7.5, showed peaks at 377, 397, and 418 nm, characteristic of the monomer emission of the pyrenyl moiety. However, when the adduct was incubated under the same conditions used in the tryptic digestion (50 mM NH_4HCO_3, pH 8.5), an additional peak at 388 nm appeared with the simultaneous decrease of the peak at 377 nm. These fluorescence changes can be assigned to different structural forms of PyM originating as a consequence of partial succinimido ring cleavage (Liburdy, 1978; Ishii & Lehrer, 1986). Therefore, the hydrolysis of the succinimido ring during the tryptic digestion procedure of the labeled enzyme samples must lead to peptides of different elution times. Greasley et al. (1994) have recently reported that PyM-modified peptides from bovine inositol monophosphatase were also resolved into two components in reverse-phase HPLC.

In previous communications we reported the specific labeling of Cys^{365} with 1,5-IAEDANS (Encinas et al., 1990; Alvear et al., 1992). On the other hand, Cys^{458} could not be independently labeled in the wild-type enzyme, because it reacts much slower than the former with 1,5-IAEDANS and IANBD (Alvear et al., 1994) and at comparable rates with PyIAM (Rojas et al., 1993). Amino acid analysis of the PyM-labeled peptides demonstrated that two different residues in the native yeast PEP carboxykinase were labeled and that they correspond to the same vicinal sulfhydryl groups evidenced when the protein was reacted with PyIA (Rojas et al., 1993). However, in the latter case the binding stoichiometry of 2 mol of PyIA/mol of enzyme subunit and the presence of an intense pyrene excimer fluorescence provided evidence for the reaction of the probe with two spatially close sulfhydryl residues. In the present experiments, the absence of excimer pyrene fluorescence (see below) and the 1:1 stoichiometry found for the labeling with PyM can be easily understood in terms of the structural characteristics of the alkylating moiety bound to the pyrene chromophore. A maleimide ring bound to pyrene must confer less conformational freedom to the molecule than an acetamide chain, which is more flexible, and thus the reaction of two PyM molecules would be hindered, giving mutually exclusive binding.

Reports on the mutually exclusive binding of probes to proteins are not frequent in the literature, although some examples have been communicated such as the *N*-ethylmaleimide labeling of Ca^{2+}-ATPase (Saito-Nakatsuka et al., 1987) and the binding of fluorescein isothiocyanate to Na,K-activated ATPase (Xu, 1989). More recently, Kazuta et al. (1991) have reported the modification of five lysyl residues in a mutually exclusive way in potato tuber UDP-glucose pyrophosphorylase modified by uridine diphosphopyridoxal.

Fluorescence Studies of the PyM-Labeled PEP Carboxykinases. Microenvironment Characteristics of Cys^{365} and Cys^{457}. The fluorescence spectra of the pyrene chromophore were similar for the native and the mutant enzymes, showing the pyrene monomer structured band in the region of 380−440 nm, while no excimer band at 470 nm was observed.

The fluorescence characteristics of the excited pyrene chromophore are highly sensitive to the local environment conditions (Encinas & Lissi, 1985; Encinas et al., 1994), so that they can provide information on the microenvironment around the probe. Pyrene singlet lifetime is considerably shortened by the presence of water. Besides, local distribution of the amino acid residues around the probe will control the excited-state lifetime. The pyrene singlet is effectively quenched by tryptophan (Encinas & Lissi, 1986). Lysyl and arginyl residues have been also mentioned as quenching agents of pyrene (Desie et al., 1987). We found that quenching of the model compound PyM-Ac by tryptophan follows a similar pathway, and that it is also quenched by histidine and methionine, with k_q values of 12×10^8 and 7×10^8 M^{-1} s^{-1}, respectively.

The fact that only a single cysteinyl residue was labeled in the mutant enzymes allowed the independent analysis of

Table 3: Fluorescence Lifetimes of the PyM-Labeled *S. cerevisiae* PEP Carboxykinases and of PyM–*N*-Acetylcysteine

PyM adduct	media	τ_1 (ns)	τ_2 (ns)
native enzyme	buffer[a]	79 (0.51)[b]	19 (0.44)
C365S mutant	buffer	98 (0.53)	27 (0.43)
C458S mutant	buffer	85 (0.52)	24 (0.44)
N-acetylcysteine[c]	water	5.5	
N-acetylcysteine[c]	ethanol:water[d]	31.5	
N-acetylcysteine[c]	ethanol	81.5	

[a] 50 mM potassium phosphate buffer, pH 7.2. [b] Values in parentheses are the relative emission contribution of each component. [c] Fluorescence lifetimes were measured under a nitrogen atmosphere. [d] 1:1 (v:v).

the fluorescence characteristics of the pyrene chromophore attached to each residue. The pyrene fluorescence decays for the native and both mutant labeled carboxykinases were well described by a double-exponential function with a long- and a short-lived component of similar amplitude (Table 3). These results show a similar heterogeneity for the three proteins, although a larger value for the long-lived lifetime decay can be observed when the fluorophore is bound to Cys^{458}. The fluorescence of the model compound was examined in different solvents. In all media, the fluorescence decay fitted to a single-exponential function with a lifetime markedly dependent on the solvent properties (Table 3). The fact that the fluorescence of PyM-Ac presents a single-exponential lifetime whereas the emission of the pyrenyl chromophore bound to the enzymes is described by two components, independent of the presence of one or two labeled residues, indicates that the heterogeneity is an intrinsic property of the enzyme-bound fluorophore. Heterogeneous emissions are frequently found in fluorophores bound to a single site of a protein (Lakowicz & Cherek, 1981), and they are usually interpreted in terms of different microenvironments sensed by the probe during its lifetime.

Studies of solvent accessibility to the pyrene probe covalently attached to PEP carboxykinases were carried out using neutral (acrylamide) and ionic (NaI) quenchers. The emission intensity and lifetimes of pyrene bound to the native and C458S mutant enzymes remained almost constant up to 1 M acrylamide. However, a decrease of ~15% at 1 M acrylamide could be observed when the pyrenyl moiety was bound to Cys^{458} in the C365S mutant protein. The activity of the enzymes remained constant in this range of acrylamide concentration. Quenching experiments carried out on PyM-Ac showed that the quenching rate constant by acrylamide is 3.6×10^8 M^{-1} s^{-1} with water as solvent, and it decreases to 1.3×10^7 M^{-1} s^{-1} in water:ethanol (1:1). This drastic dependence of the rate on the solvent polarity is usually found for processes that take place through a charge-transfer mechanism (Encinas et al., 1983; Encinas & Lissi, 1985). Therefore, the absence of quenching by acrylamide for protein-bound pyrene can be due to the inaccessibility of the quencher to the probe sites or to a low polarity of the microenvironments where the pyrene–acrylamine encounter takes place. It is difficult to separate the effect of microenvironmental polarity from that of inaccessibility. However, the difference in quenching efficiency for the chromophore bound to the mutant enzymes suggests somewhat different microenvironments for the pyrenyl groups bound to Cys^{458} and Cys^{365}.

The fluorescence quenching by iodide of the model compound PyM-Ac shows a normal behavior. The Stern–

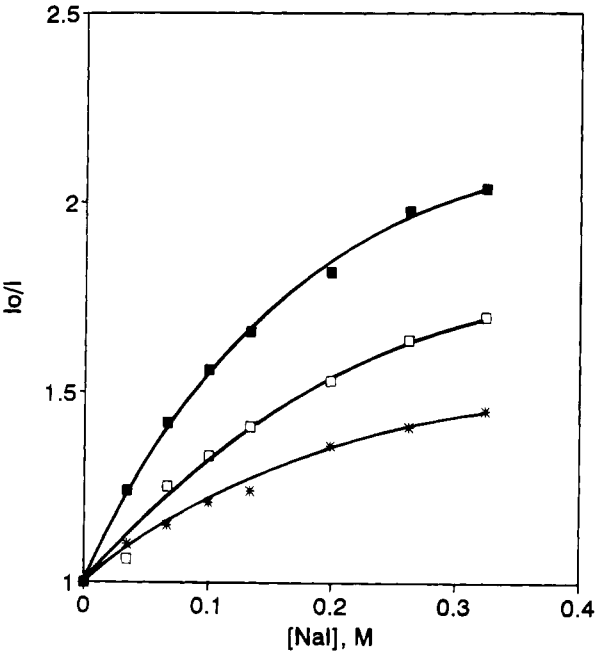

FIGURE 4: Fluorescence quenching of the PyM-labeled *S. cerevisiae* PEP carboxykinases. Stern–Volmer plots of the pyrene fluorescence intensity quenching by NaI for the wild-type (□), C365S (■), and C458S (*) enzymes in 50 mM phosphate buffer, pH 7.2 at 25 °C. Excitation was 340 nm, and emission was 400 nm.

Volmer plots, either in $I°/I$ or $\tau°/\tau$, are linear with similar slopes, indicating the predominance of dynamic quenching. A limited diffusion-controlled value of 4×10^9 M^{-1} s^{-1} was obtained for the quenching rate constant, in water at pH 7.5. The emission quenching of pyrene bound to the proteins showed a different behavior. The quenching of the steady-state fluorescence gave downward curved Stern–Volmer plots, the quenching efficiency being markedly different for the three proteins (Figure 4). The downward curvature of Stern–Volmer plots can be explained in terms of the heterogeneous population of the fluorophores, which differ significantly in their individual susceptibility to the ionic quencher. The decrease of pyrene fluorescence by iodide addition is considerably higher for the chromophore bound to Cys^{458} than to Cys^{365}, pointing to different accesibility of the quencher to the probe when bound to one or the other residue.

Phase and modulation measurements were carried out in the presence of increasing NaI concentrations. These experiments also showed a quite different fluorescence behavior of the probe depending upon whether it was attached to Cys^{365} or Cys^{458}. Emission lifetimes for the probe bound to Cys^{365} were almost unchanged by the addition of quencher. A decrease of 15% on the longer lifetime component could be observed at an iodide concentration of 0.36 M, while the shorter lifetime component appeared as unaltered. Therefore, the higher observed quenching in steady-state experiments must be due to some contribution of static quenching. In the case of pyrene bound to Cys^{458} the data clearly indicate a dependence of both lifetime components on the quencher concentration. From the Stern–Volmer plots, rate constant values of 3×10^7 and 1×10^7 M^{-1} s^{-1} were calculated for the short- and long-lived components, respectively. Experiments carried out with NaCl showed that the intensity or decay of the emission of the pyrenyl group bound to the proteins did not change with addition of salt up to 1 M. These

results rule out any ionic strength induced alteration in the protein conformation due to the presence of iodide.

The heterogeneity of the time-resolved fluorescence of pyrene bound to the mutant enzymes presents subtle differences; these results together with those of the fluorescence quenching studies suggest differences in the topology of the two cysteines region. Thus, the fluorescence quenching by NaI indicates that Cys^{458} is much more accessible to the ionic compound than Cys^{365}. Acrylamide quenching experiments, though they are less conclusive, point in the same direction. Then, Cys^{458} has to be located in a region somewhat accessible to the solvent, while Cys^{365} appears to be located more deeply in the protein matrix.

In conclusion, we have demonstrated that the reactive Cys^{365} and Cys^{458} residues of *S. cerevisiae* PEP carboxykinase are of no catalytic relevance, a finding that could probably be extended to the highly reactive cysteinyl residues reported in other PEP carboxykinases. The obtainment of the mutated C365S and C458S enzymes allowed the characterization of the protein topology around these residues, which are apparently located in the region of the nucleotide binding site.

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