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AFFINITY LABELING OF PHOSPHOENOLPYRUVATE CARBOXYKINASE WITH 1,5-I-AEDANS

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SUMMARY: Phosphoenolpyruvate carboxykinase, EC 4.1.1.32, is affinity labeled by N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (1,5-I-AEDANS) with saturation kinetics of inactivation and specific substrate protection. Approximately 1.3 moles of dansyl label are incorporated per mole of enzyme inactivated. The specificity of the enzyme modification, with retention of fluorescent label, was evident upon polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate, and upon mapping and gel filtration of its tryptic peptides. Fluorescence emission from the modified enzyme suggests the label to be located in an environment of reduced polarity.

Phosphoenolpyruvate carboxykinase (PEPCK) is inactivated by several reagents including N-ethylmaleimide (1) and bromopyruvate (2), with demonstrated cysteine residue modification. Recently, DTNB has been shown to be particularly reactive towards a single sulfhydryl group in PEPCK, that suggested hydrophobic interaction of DTNB with the enzyme. This sulfhydryl was shown to be vicinal to a second sulfhydryl (3). 1,5-I-AEDANS is a reagent introduced by Hudson and Weber (4). It contains the dansyl group and exhibits a visible fluorescence emission maximum that is blue-shifted in environments of reduced polarity. It also contains the iodoacetamido group and thus is a sulfhydryl reagent (4).

MATERIALS AND METHODS: Hog liver mitochondrial and cytosol PEPCK were purified to homogeneity as judged by sodium dodecylsulfate polyacrylamide gel electrophoresis. The specific activity of the pure enzymes were 24 (mitochondrial) and 19 (cytosol) μmol $min^{-1}mg^{-1}$. Purification was achieved in each instance by salt-mediated hydrophobic chromatography and affinity chromatography from initial broad ammonium sulfate cuts (5,6). PEPCK activity was assayed at 37° in 1 ml reaction mixtures that contained 110 μmol imidazole-chloride, pH 5.8, 5 μmol $MnCl_2$, 0.10-0.15 μmol NADH, 0.5 μmol IDP, 0.6 units malate dehydrogenase from beef heart (Boehringer-Mannheim). After a 3 min preincubation, 75 μmol of $NaHCO_3$ were added at 37° and PEPCK activity monitored at 340 nm on a recording spectrophotometer.

Abbreviations: 1,8-ANS, 1-anilinonaphthalene-8-sulfonate; DTNB, 5,5'-dithiobis (2-nitrobenzoate); 1,5-I-AEDANS, N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

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1,5-I-AEDANS was obtained from Molecular Probes, and stored desiccated in the dark at -20°. 0.025M solutions of 1,5-I-AEDANS in 0.55M imidazole-chloride, pH 6.1, were prepared fresh daily. Imidazole of low flourescence blank, was obtained from Sigma Chemical Co.

Inactivation kinetics - 1,5-I-AEDANS was added to incubation mixtures of PEPCK in 110 mM imidazole at pH 6.1, temperature, 37°, with aliquots removed at different times to be assayed for residual PEPCK activity. The PEPCK solutions included 140 mM 2-mercaptoethanol to insure effective quenching. Direct addition of 1,5-I-AEDANS to such assay solutions containing PEPCK did not lead to inactivation, nor did the addition of 1,5-I-AEDANS to incubation mixtures that contained 140 mM mercaptoethanol. Apparent first-order rate constants were determined from least squares analysis of log residual activity vs. time. Data were obtained with the mitochondrial PEPCK, unless otherwise indicated.

Isolation and characterization of the modified enzyme - 1,5-I-AEDANS was added to two solutions of PEPCK, one containing 5×10^{-3}M GDP, 1×10^{-2}M PEP, 1×10^{-2}M Mn^{2+}, and the other without added protecting agents. Otherwise, conditions for the two incubations were the same: 1.5 ml volume, containing 110 mM imidazole-Cl, 2.5×10^{-3}M 1,5-I-AEDANS, hog mitochondrial PEPCK, 25.1 units, final pH 6.1, temperature, 37°. At 2 min, inactivation was quenched with 140 mM 2-mercaptoethanol and determined to be 84 and 8 percent in the respective absence and presence of protecting agents. The incubation mixtures were then each placed on a 1.5 x 40.5 cm Biogel P-10 column and eluted with 5×10^{-3}M phosphate, pH 7.0. The resulting enzyme fractions were well separated from excess dansyl reagent, and were assayed for residual activity and for fluorescence emission at 475 nm. The modified enzyme fractions were then pooled for further analysis, and the fluorescence emission spectrum recorded.

Fluorescence measurements were obtained on a Perkin-Elmer MPF-44A fluorescence spectrophotometer. Solutions were excited at 340 nm and fluorescence emission recorded between 600-300 nm. Spectra were corrected by a microprocessor based differential corrected spectra unit (Perkin-Elmer). UV absorption measurements, for stoichiometry determination, were made on a Cary 118 spectrophotometer. Polyacrylamide gel electrophoresis of the native and modified enzyme was carried out as previously described (5). Far ultraviolet CD spectra of the native and modified enzyme were obtained in 5×10^{-3}M phosphate, pH 7.0, on a Jasco J-20 spectropolarimeter.

The modified enzyme was reduced and alkylated in 8 M urea, as previously described (7). The sample was then extensively dialyzed in the dark against 0.1 per cent ammonium bicarbonate. Tryptic digestion was initiated with the addition of 2 per cent by weight of TPCK treated trypsin dissolved in 10^{-3}M HCl. The digest was maintained at 37° for 2 hours, at which time, a second equivalent trypsin addition was made. The digest was maintained at 37° for an additional 2 hours and then was terminated by adjusting the pH to 6.0 and immediately freeze drying. The lyophilized residue was subjected to peptide mapping on thin layer cellulose. A sample equivalent to approximately 0.1 mg of enzyme was dissolved in 50 per cent acetic acid and spotted on a thin layer plate. Separation in the first dimension was by chromatography using a solvent consisting of butanol:pyridine:acetic acid: water (15:10:3:12). Following chromatography, the thin layer plate was carefully dried and then sprayed with dilute pyridine:acetic acid buffer at pH 3.5 and separation carried out in the second dimension by electrophoresis in a field of 15V per cm. Upon completion of the peptide mapping, the fluorescent peptides were visualized using a hand held short wavelength UV light (mineral light).

RESULTS: Incubation of PEPCK with 1,5-I-AEDANS produced a time-dependent loss of activity that was first-order in enzyme, Fig. 1. The first-order plots

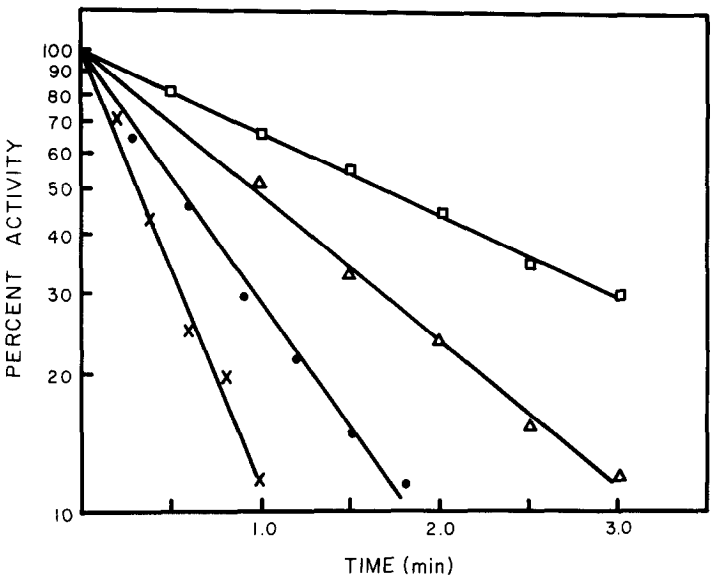

Fig. 1. Inactivation of PEPCK by 1,5-I-AEDANS. Conditions are as described in Materials and Methods. 1,5-I-AEDANS concentrations, M x 10^3, x, 2.5; ●, 1.0; △, 0.5; □, 0.25.

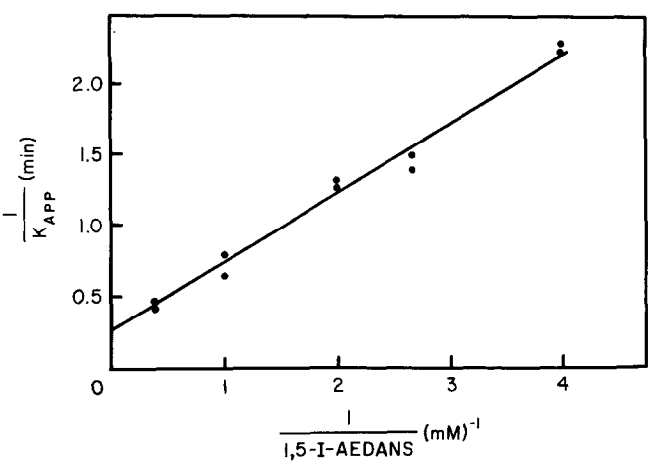

Fig. 2. Double reciprocal plot of the apparent first-order rate constants, from Fig. 1, vs. 1,5-I-AEDANS concentration.

extrapolate at zero time to 100 per cent activity, as determined by incubation in the absence of 1,5-I-AEDANS. The double reciprocal plot of apparent first-order rate constants vs. 1,5-I-AEDANS, Fig. 2, demonstrates saturation kinetics of inactivation, indicating an affinity of the enzyme for the reagent,

with binding preceding inactivation. The corresponding dissociation constant for 1,5-I-AEDANS•PEPCK complex

$$E + 1,5\text{-I-AEDANS} \rightleftarrows E\cdot(1,5\text{-I-AEDANS})$$

$$E\cdot(1,5\text{-I-AEDANS}) \rightarrow \text{inactive enzyme}$$

was calculated to be 1.82×10^{-3} M. Iodoacetamide and iodoacetate also inactivate PEPCK under comparable conditions but at a rate about one-eight that by 1,5-I-AEDANS.

The apparent first-order rate constants (kapp) for inactivation by 1,5-I-AEDANS increase with pH as follows:

pH	kapp, min^{-1}
5.95	.083
6.1	.128
6.5	.410
6.95	1.08
7.05	1.35
7.28	2.03

Conditions: 110 mM imidazole -Cl, 1.33×10^{-4} M 1, 5-I-AEDANS, temperature 37°. Comparison of the rates of mitochondrial and cytosol PEPCK inactivation by 1,5-I-AEDANS (pH 6.1, 5×10^{-4}M 1,5-I-AEDANS) gave apparent first-order rate constants of 0.59 and 0.64 min^{-1}, respectively, indicating, within experimental error, that both enzymes are inactivated at the same rate.

Substrates protect against inactivation by 1,5-I-AEDANS as follows: 2.5×10^{-4}M IDP, 77%; 5×10^{-3}M PEP, 78%; 5×10^{-3}M Mn^{2+}, 77%; 5×10^{-3}M PEP plus 5×10^{-3}M Mn^{2+}, 85%. ADP, which is not involved in catalysis protects poorly: 2.5×10^{-4}M ADP, 13%. Protection by PEP and IDP was found competitive with 1,5-I-AEDANS, with corresponding values of K_d, calculated (8) to be 1×10^{-3}M and 6.3×10^{-5}M, respectively. IDP was found to also protect against inactivation by iodoacetamide and iodoacetate.

The IAEDANS-modified enzyme was separated from the inactivation mixture upon Biogel P-10 chromatography, see Materials and Methods. Negligible fluorescence was incorporated for the incubation with 1,5-I-AEDANS and carried out in the presence of substrate protecting agents, Fig. 3. The dansyl fluorescence

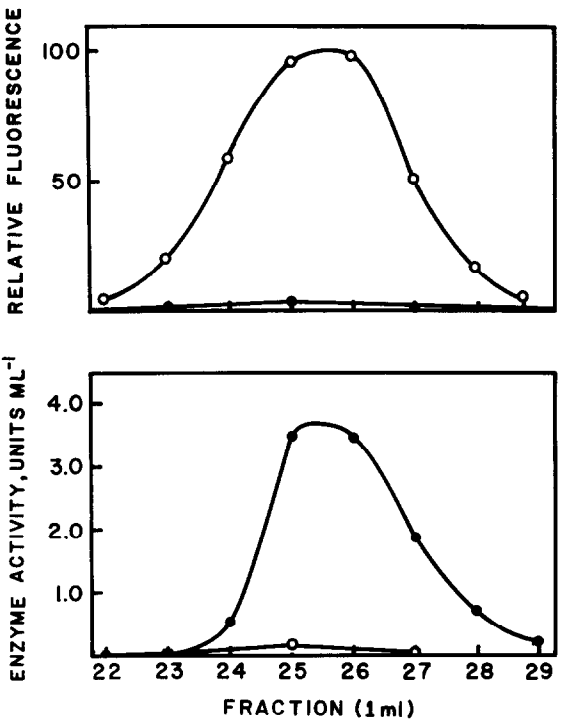

Fig. 3. Biogel P-10 chromatography elution profiles for PEPCK incubated with 2.5×10^{-3}M 1,5-I-AEDANS in the absence (o) and presence (●) of protecting agents, see Materials and Methods. Top: fluorescence incorporation; bottom: residual enzyme activity.

spectrum of the modified enzyme exhibits a maximum at 475 nm, Fig. 4, indicative of incorporation of the dansyl moiety into a microenvironment of reduced polarity (4). From the molar concentration of enzyme inactivated, the 340 nm absorbance of the modified enzyme, and the corresponding extinction coefficient for the dansyl moiety (4), it was estimated that 1.3 moles of dansyl label were incorporated per mole of enzyme inactivated. A single fluorescent band and corresponding single protein band (Coomassie blue staining) was apparent upon polyacrylamide gel electrophoresis of the modified enzyme in the presence of sodium dodecylsulfate.

The CD spectrum of the modified enzyme, recorded between 250-200 nm, was indistinguishable from that of the native enzyme. The per cent α-helix, calculated from the mean residue weight ellipticity at 222 nm (9) was 14.3 per cent

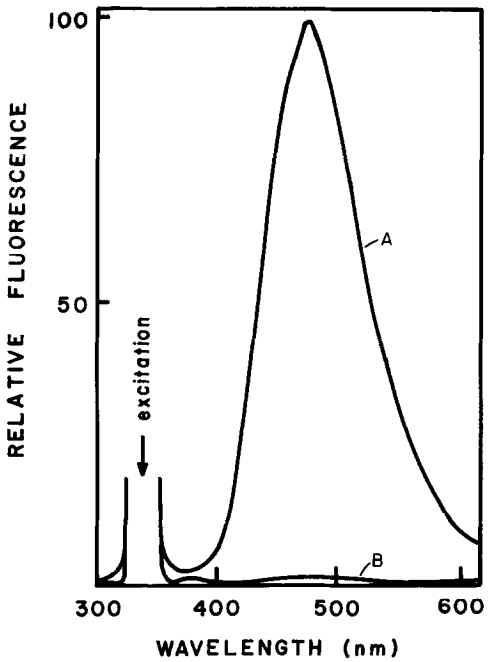

Fig. 4. Fluorescence emission spectra (true) of eluted enzyme pools from Fig. 3. A, incubation without protecting agents; B, incubation with protecting agents.

before and 14.4 per cent after modification. The mean residue weight ellipticity at 208 nm (10) gave corresponding α-helix contents of 8.4 per cent and 8.7 per cent, respectively.

Peptide mapping after reduction and alkylation in the presence of 8 M urea followed by extensive dialysis and tryptic digestion gave a single fluorescent spot, see Materials and Methods. Fractionation of the tryptic peptides on Biogel P-30 chromatography gave a single fluorescent peak.

DISCUSSION: The saturation kinetics of PEPCK inactivation by 1,5-I-AEDANS, the ability of substrates to protect against inactivation, and the retention of the fluorescent label under conditions expected to release non-covalently bound molecules, indicate that the enzyme has been affinity labeled (11). The ability of substrates to block the incorporation of label argues against significant nonspecific enzyme labeling, as do the tryptic peptide findings, and the approximate 1:1 modification stoichiometry.

Inactivation of PEPCK by IAEDANS might conceivably arise not only from IAEDANS iodoacetamido reactivity with an enzyme sulfhydryl or other enzyme nucleophile[1], but also from noncovalent interaction of the IAEDANS dansyl group with the enzyme. The latter possibility is of more than theoretical interest, particularly in view of our finding that 1,8-ANS also inactivates PEPCK and without covalent modification. Inactivation by ANS also proceeds with saturating kinetics of inactivation (K_i = 1.7 x 10^{-4}M, imidazole buffer, pH 5.8) and with substrate protection against both inactivation (5 mM PEP, 91%; 0.5 mM IDP, 99%) and the appearance of fluorescence due to PEPCK bound ANS (4.5 mM PEP, 100%; 0.33 mM IDP, 83%) (12). It is therefore conceivable that the labeling of PEPCK by IAEDANS, subsequent to binding, may be incidental to, rather than a requirement for, inactivation.

To distinguish between these possibilities, the effect of pH on the rate of inactivation is instructive. With IAEDANS, there is a marked increase in inactivation rate upon increasing the pH from slightly acid to slightly base, see Results. Such an effect was also observed previously upon inactivation of PEPCK by N-ethylmaleimide, which does not involve preliminary binding (kinetics are not saturating) and which results in cysteinyl modification (1). Similar inactivation kinetics, including the effect of pH, have likewise been observed upon inactivation of PEPCK by bromopyruvate (2). Recently, using [3-^{14}C]bromopyruvate (13), specific cysteinyl labeling has been demonstrated in this instance also, and with complete substrate (GDP) protection against the inactivation and the incorporation of the isotopic label (14).

The rate of PEPCK inactivation by 1,8-ANS, in contrast, decreases with increasing pH, from an apparent first-order rate constant of 2.28 min^{-1} at pH 5.85 to 0.57 min^{-1} at pH 7.2 (imidazole buffer, 2.6 x 10^{-4}M ANS, 37°). A parallel decrease is seen in the fluorescence due to PEPCK bound ANS (12).

[1] Alkylation of PEPCK by IAEDANS would be expected to result in iodide release. This, however, does not appear to itself result in inactivation as 2.5 mM NaI neither inhibits nor inactivates PEPCK under conditions of inactivation by IAEDANS.

It would appear, therefore, that, with IAEDANS, both the binding and the resulting specific labeling are important elements of the inactivation process. The specificity of the labeling should prove useful in elucidating structural features of PEPCK related to its catalytic properties and the stability of its active-site.

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REFERENCES

1. Barnes, R.J. and Keech, D.B. (1968) Biochim. Biophys. Acta 159, 514-526.
2. Silverstein, R. (1976) Fed. Proc. 35, 1756.
3. Carlson, G.M., Colombo, G., and Lardy, H.A. (1978) Biochemistry 17, 5329-5338.
4. Hudson, E.N. and Weber, G. (1973) Biochemistry 12, 4154-4161.
5. Hung, B.T. and Silverstein, R., Prep. Biochem., in press.
6. Hung, B.T. and Silverstein, R. (1978) Fed. Proc. 37, 1427.
7. Crestfield, A.M., Moore, S., and Stein, W.H. (1963) J. Biol. Chem. 238, 622-627.
8. Kitz, R. and Wilson, I.B. (1962) J. Biol. Chem. 237, 3245-3249.
9. Chen, Y.H., Yang, J.T., and Martinez, H.M. (1972) Biochemistry 11, 4120-4131.
10. Greenfield, N. and Fasman, G.D. (1969) Biochemistry 8, 4108-4115.
11. Wold, F. (1977) in Methods in Enzymol. (Jakoby, W.B. and Wilchek, M., eds) Vol 46, pp 3-14, Academic Press, N.Y.
12. Lin, C.C., Rawitch, A.B., and Silverstein, R., in preparation.
13. Meloche, H.P. (1970) Biochemistry 9, 5050-5055.
14. Silverstein, R., unpublished results.