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INVOLVEMENT OF TYROSYL RESIDUES IN THE SUBSTRATE BINDING OF PIGEON LIVER MALIC ENZYME

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Summary

The reactions of pigeon liver malic enzyme (L-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40) with tetranitromethane and *N*-acetylimidazole have been investigated to obtain information about the functional role of tyrosine residues in this enzyme. Incubation of the sulfhydryl-masked enzyme with tetranitromethane or *N*-acetylimidazole caused a time-dependent loss of all enzymatic activities of this enzyme. The absorption spectra of both the nitrated and acetylated enzyme indicated modification of tyrosine residues. The enzymatic activity of the acetylated enzyme was reversed by hydroxylamine. No amino group modification was observed. Preincubation of the enzyme with dicarboxylate substrate (or inhibitor), nucleotide coenzyme and divalent metal ions protected the enzyme against these reagents. The acetylated enzyme showed different kinetic properties from the native enzyme. The apparent Michaelis constants for malate and oxaloacetate increase by 2—5-fold. The binding between acetylated enzyme and NADPH was not abolished. These results strongly suggest the involvement of tyrosine residues in the dicarboxylic acid binding of malic enzyme.

Introduction

Malic enzyme (L-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40) catalyzes the reversible oxidative decarboxylation of malate to CO_2 and pyruvate (M^{2+} = divalent metal ion):

$$\text{Malate} + NADP^+ \overset{M^{2+}}{\rightleftharpoons} CO_2 + \text{pyruvate} + NADPH + H^+ \quad (1)$$

This reaction occurs without detectable intermediates, but is resolvable into the decarboxylase and reductase partial reactions [1—5]:

$$\text{Oxaloacetate} \xrightarrow[(\text{pH } 4.5)]{M^{2+}} CO_2 + \text{pyruvate} \quad (2)$$

$$\alpha\text{-Keto acid} + \text{NADPH} + H^+ \xrightarrow{M^{2+}} \alpha\text{-hydroxy acid} + \text{NADP}^+ \quad (3)$$

Pigeon liver malic enzyme has a molecular weight of 260 000 and is a tetramer composed of probably identical subunits [6,7]. Chemical modification was chosen as a probe in an attempt to identify the essential amino acid residues in the active center of this enzyme and to characterize their role in the catalytic mechanism [8—13]. The inactivation of malic enzyme by sulfhydryl reagents indicated that the SH-group plays a structural, rather than a catalytic role [8]. On the other hand, inactivation of the enzyme by ethoxyformic anhydride indicated the involvement of histidyl residues in the nucleotide binding [11]. More recently, we found oxidized NADP to be an affinity label of this enzyme [13]. In the presence communication, we wish to report the inactivation of pigeon liver malic enzyme by two tyrosine-specific reagents tetranitromethane and *N*-acetylimidazole. Our results suggest that tyrosyl residues are involved in the substrate binding of this enzyme. A preliminary report has been presented [14].

Materials and Methods

Materials. L-Malic acid, sodium pyruvate, oxaloacetate, dithioerythreitol, *p*-chloromercuribenzoate, 5,5′-dithiobis-(2-nitrobenzoic acid), *N*-acetylimidazole, NADP, NADPH (Sigma); hydroxylamine-HCl (recrystallized with deionized water) (Matheson); tartronate, tetranitromethane (Wako) were purchased from the designated sources. All other chemicals were of reagent grade or better. Distilled deionized water was used.

Pigeon liver malic enzyme was purified according to Hsu and Lardy [15]. Each preparation was routinely checked for high specific activity, and analyzed for purity by acrylamide gel electrophoresis [16]. Protein concentration was determined spectrophotometrically at 278 nm, using an extinction coefficient of 0.86 for a 0.1% (w/v) protein solution [15].

Enzyme assays. Oxidative decarboxylase activity was assayed at 30°C according to Hsu and Lardy [15]. The formation of NADPH was monitored at 340 nm. The reductase activity was assayed according to Tang and Hsu [8], using pyruvate as substrate. The disappearance of NADPH was monitored at 340 nm and 30°C. The oxaloacetate decarboxylase activity was assayed essentially according to Kosicki [17] by following the disappearance of oxaloacetate at 260 nm and 24°C (cf. Ref. 10).

Preparation of sulfhydryl-masked enzyme. In the routine preparation of sulfhydryl-masked enzyme, malic enzyme (0.57 μM) was incubated with 60 μM *p*-chloromercuribenzoate in borate buffer (pH 7.5) at room temperature for 15 min. Usually, no enzyme activity was left after such treatment. The

enzyme activity could be fully recovered by treating the modified enzyme with excess dithioerythreitol (25 mM) for 5 min.

Modification of malic enzyme with tetranitromethane. Tetranitromethane was dissolved in absolute ethanol just before use. Modification experiments were performed at 24°C by the addition of tetranitromethane to the sulfhydryl-masked enzyme in Tris-HCl buffer at pH 8.0. The progess of the reaction was monitored by assaying the oxidative decarboxylase, reductase, or oxaloacetate decarboxylase activities on small samples withdrawn at designated time intervals after the sulfhydryl groups were recovered by dithioerythreitol. The kinetics of inactivation were examined by plotting the natural logarithm of residual activity against time.

Modification of malic enzyme with N-acetylimidazole. *N*-Acetylimidazole was dissolved in 50 mM borate buffer (pH 7.5) just before use. Modification procedures were the same as tetranitromethane modification besides using different buffer. The amount of *N*-acetylimidazole introduced by each enzyme sample for assay was small and insufficient to cause further inactivation.

Spectral analysis. After nitration, ultraviolet difference spectrum was determined before and after gel filtration through Sephadex G-25 or extraction with tributylphosphate [18] to remove the brilliant yellow substance nitroformate.

Difference spectrum of the acetylated-enzyme was performed as above but without removing any molecule. *N*-Acetylimidazole was added to the reference and sample cuvettes simultaneously. The absorbance decrease at 278 nm was monitored versus time. Difference spectra were taken during the reaction course and after hydroxylamine treatment.

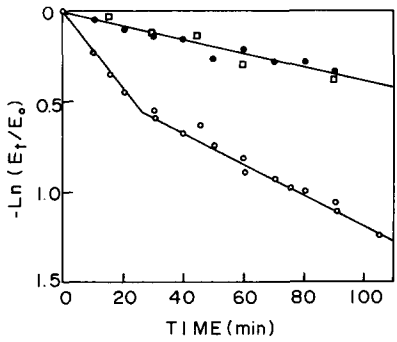
Fluorescence titration. Fluorescence titration of the malic enzyme with NADPH was performed using an Aminco-Bawman spectrofluorimeter. The nucleotide was excited at 350 nm and the emission fluorescence at 450 nm was monitored [6]. In the control experiment, all reagents except the enzyme were added for correction of the quenching due to reagents.

Results and Discussion

Our preliminary experiments showed that the native enzyme was inactivated rapidly by the tyrosine-specific reagents tetranitromethane and *N*-acetylimidazole. Titrating the modified enzyme with 5,5′-dithiobis-(2-nitrobenzoic acid) showed extensive loss of sulfhydryl groups, indicating that SH-groups were oxidized or acetylated simultaneously with the nitration or acetylation of tyrosine residues. Since SH-group is essential for malic enzyme activity [8,19], it became necessary to protect these groups before further modification experiments.

Alteration of the malic enzyme activities by tetranitromethane

The reaction of the SH-masked malic enzyme with tetranitromethane caused an irreversible loss of the oxidative decarboxylase activity. Both partial activities, the reductase and oxaloacetate decarboxylase activities were decreased. The pseudo first-order plot for the oxidative decarboxylase inactivation followed biphasic kinetics (Fig. 1). First, there was a rapid reaction until about 50% inactivation was reached. The remaining activity was then lost at a



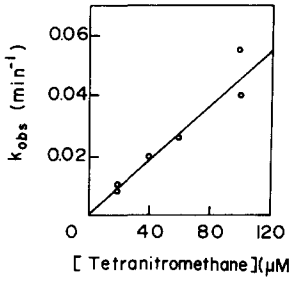


Fig. 1. The alteration of intrinsic activities of malic enzyme by tetranitromethane. Tetranitromethane was added to the SH-masked enzyme (0.53 μM) to give a final concentration of 69 μM for tetranitromethane and 0.77% for ethanol. At designated time intervals, aliquots (25 μl) were withdrawn and excess dithioerythreitol (25 mM) was added to restore the SH-groups. After 5 min incubation, the activities were assayed. (○), oxidative decarboxylase; (●), reductase; (□), oxaloacetate decarboxylase. It was shown in the control experiment that ethanol up to 4.5% did not inactivate the enzyme.

Fig. 2. The effect of tetranitromethane concentrations on the rate of oxidative decarboxylase inactivation. Conditions for inactivation were as described in Fig. 1, except that tetranitromethane concentrations were varied as indicated. The pseudo first-order constant for the first inactivation phase were used.

slower rate. The pseudo first-order rate constants calculated from the slopes of these two lines were 0.02 min^{-1} and 0.0082 min^{-1}, respectively. On the other hand, the decrease of reductase and decarboxylase activities followed simple pseudo first-order kinetics. The inactivation rate constants were both 0.004 min^{-1}.

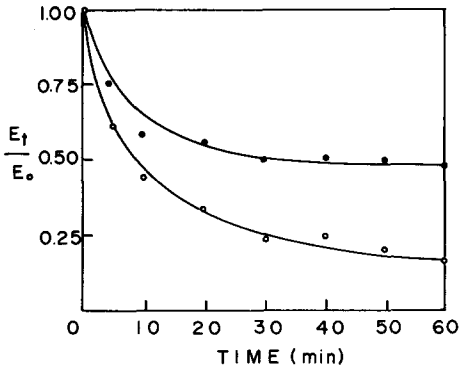
The rate of oxidative decarboxylase activity loss was highly dependent upon tetranitromethane concentration. When the observed inactivation rates of the first phase modification were plotted against tetranitromethane concentrations, a straight line going through the origin was obtained (Fig. 2). This indicated that no reversible enzyme-inhibitor intermediate complex formed prior to irreversible modification. A plot of log k_{obs} against log[tetranitromethane] yield a straight line with a slope of 1.07, suggesting that one molecule of tetranitromethane per enzyme subunit was needed for the rapid inactivation of the enzyme [20].

Protection by oxaloacetate against tetranitromethane inactivation

Protection of the enzyme against tetranitromethane inactivation was afforded by oxaloacetate together with NADP and Mn^{2+} (Fig. 3). Inhibitors or other substrates plus coenzyme and divalent metal ions gave similar but less protection (data not shown). These results suggested that the inactivation might be in or near the active center.

Spectral analysis and effect of pH on the tetranitromethane modification

Ultraviolet difference spectrum of the tetranitromethane-modified enzyme versus SH-masked enzyme showed a sharp absorption maximum at 350 nm, characteristic for the nitroformate [21]. After removing the latter by gel filtration or tributylphosphate extraction [18], the modified enzyme showed an



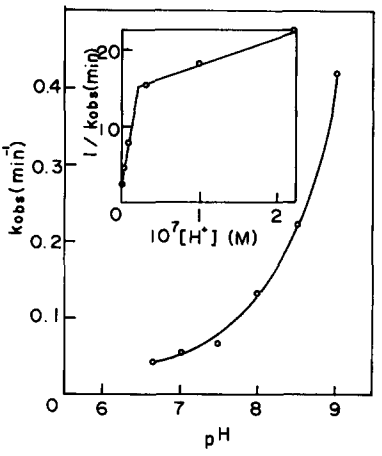


Fig. 3. Protection of the sulfhydryl-masked enzyme against tetranitromethane inactivation. Sulfhydryl-masked enzyme (0.42 μM) was incubated with 84 μM tetranitromethane in 58 mM Tris-HCl buffer (pH 8.0) at 24°C with (•) or without (○) the following addition: 2.7 mM oxaloacetate, 75 μM NADP, and 1.3 mM Mn^{2+}.

Fig. 4. The effect of pH on tetranitromethane inactivation. The conditions of this experiment were as described in Fig. 1, except that Tris-HCl buffer was adjusted to the desired pH values and tetranitromethane concentration was 0.31 mM. The enzyme was fully active without tetranitromethane in the pH range studied.

absorption peak at 428 nm, characteristic for the nitrotyrosyl residue [21]. The 428 nm peak disappeared after reducing with sodium hydrosulfite.

The effect of pH on the inactivation rate was investigated in order to estimate the pK value of the essential groups being modified. The plot of k_{obs} versus pH (Fig. 4) was further analyzed by plotting $1/k_{obs}$ against [H^+] [22], which gave two straight lines (inset). Extrapolation gave ionization constants (K) of $3.8 \cdot 10^{-7}$ and $5 \cdot 10^{-9}$, corresponding to pK values of 7.6 and 9.7, respectively. Since small amount of residual enzyme activity (3%) remained in the sulfhydryl-masked enzyme, SH-group was assumed to be responsible for the lower pK value. This value was not far from our previous result obtained from bromopyruvate work for SH-group (pK 8.3) [10]. The pK value 9.7 was consistant with the known value for tyrosyl residue [23].

The above results suggested that modification of tyrosyl residue by tetranitromethane was the cause of inactivation [24]. However, it should be pointed out that during dialysis of the nitrated-enzyme some insoluble material appeared. This is a common phenomenon of tyrosine modification by tetranitromethane [25—28], possibly because of cross-linking of the nitrated enzyme. Although the crosslinking reaction gave another criterion for the tyrosine modification, it made further quantitation difficult. Therefore we shifted to another tyrosine-specific reagent, N-acetylimidazole.

Alteration of malic enzyme activities by N-acetylimidazole modification

When SH-masked enzyme was treated with N-acetylimidazole, the results were similar with those obtained with the tetranitromethane modification. Incubation of the SH-masked enzyme with N-acetylimidazole at pH 7.5 caused

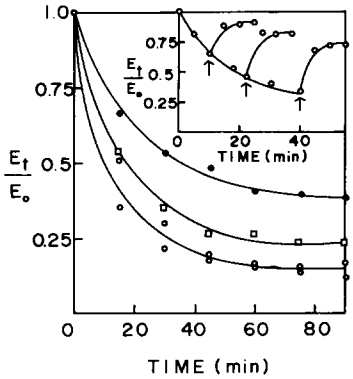


Fig. 5. The alteration of intrinsic activities of malic enzyme by *N*-acetylimidazole. The oxidative decarboxylase (○), reductase (●), and oxaloacetate decarboxylase (□) activities of this enzyme were examined. The reaction mixtures consisted of: 0.28 μM SH-masked enzyme, 2.5 mM *N*-acetylimidazole, and 50 mM borate buffer (pH 7.5). Inset, separate experiments show the reversal of *N*-acetylimidazole-caused inactivation by hydroxylamine. Samples were withdrawn from the reaction mixture at the time intervals indicated by arrows and treated immediately with 1 M NH_2OH (pH 7.5). Aliquots were removed and oxidative decarboxylase determined after the SH-groups were recovered by dithioerythreitol.

a loss of the oxdative decarboxylase, reductase, and oxaloacetate decarboxylase activities (Fig. 5). At 2.5 mM *N*-acetylimidazole and 0.28 μM malic enzyme, about 80% of the oxidative decarboxylase activity was lost in 80 min. The remaining activity could be abolished by further addition of the reagent. Higher initial *N*-acetylimidazole concentration caused faster inactivation. The inset of Fig. 5 showed the reversibility of the *N*-acetylimidazole modification. At various stages of enzyme inactivation, addition of high concentration (1 M) of hydroxylamine to the reaction mixture led to a rapid reversal of enzyme activity.

Besides the SH-groups, other functional groups in the protein side chain most likely to be affected by *N*-acetylimidazole are the amino groups. However, acetylation of amino groups in malic enzyme may be ruled out by two grounds. First, the amide linkage is stable and modification of amino groups should be irreversible (cf. Ref. 30). Secondly, when 94% of the enzyme activity was destroyed by acetylation, no decrease in titratable amino groups by sodium trinitrobenzene sulfonate was detectable.

Under certain circumstances *N*-acetylimidazole can acylate histidyl residues, but *N*-acetylhistidine is very unstable. Since the loss of activity observed on acetylation of malic enzyme with *N*-acetylimidazole was stable until hydroxylamine was added, it seems clear that the effects were due to modification of tyrosyl residues [29].

Protection by substrates or inhibitors against N-acetylimidazole inactivation

The effects of substrates or inhibitors on the inactivation of malic enzyme were studied. Substrate, coenzyme, or inhibitor alone gave little or no protection. However, substrate or inhibitor plus coenzyme gave significant protection, in accordance with the sequential kinetic mechanism with NADP as the leading reactant [31]. The results also indicate that only dicarboxylic acid (oxalo-

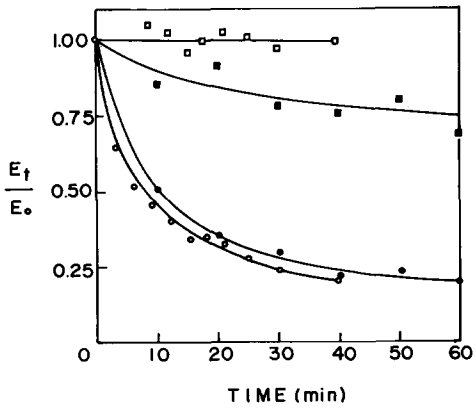


Fig. 6. Protection of the sulfhydryl-masked enzyme against *N*-acetylimidazole inactivation. SH-masked enzyme, 1.76 μM (○, □) or 0.45 μM (●, ■), was incubated with 8.1 mM (○, □) or 2.5 mM (●, ■) *N*-acetylimidazole in 50 mM borate buffer (pH 7.5) with (□, ■) or without (○, ●) the following additions: (□), 0.9 mM oxaloacetate (pH 7.5), 3.6 mM Mn^{2+}, 0.2 mM NADP; (■), 1.67 mM tartronate (pH 7.5), 0.67 mM Mn^{2+}, and 37.8 μM NADP.

acetate, tartronate, oxalate, or malate) gave substantial protection. The monocarboxylic acid pyruvate gave little protection. Oxaloacetate was the only substrate giving considerable protection without coenzyme or metal ions. Full protection was afforded by oxaloacetate plus NADP and Mn^{2+}. The noncompetitive inhibitor tartronate [10] plus nucleotide and metal also gave substantial protection (Fig. 6). This led us to believe that the dicarboxylic acid protected the enzyme by interacting at the substrate binding site. The protection against nitration was not so obvious as acetylation, possibly due to cross-linking reaction even at early stage of inactivation when no visible precipitation was detectable.

Correlation of the extent of acetylation and changes in enzymatic activity

O-Acetylation of tyrosine results in a decrease in the ultraviolet absorption of malic enzyme. The number of tyrosine residues modified was calculated using an extinction coefficient of 1160 for *O*-acetyltyrosine [30]. Pigeon liver malic enzyme has a total of 78 tyrosine residues [7]. A plot of the residual oxidative decarboxylase activity versus the number of tyrosyl residues modified by this reagent was linear (data not shown). Extrapolation of the line to complete inactivation gave 32 reactive tyrosyl residues per mol (or 8 residues per subunit). Hydroxylamine restored the original absorbance. The addition of tartronate, Mn^{2+}, and NADP reduced the extent of activity loss as well as the tyrosyl groups modified. When the activity protected was plotted versus the tyrosyl groups protected and extrapolated to 100% protection, 20 residues per mol (or 5 residues per subunit) was obtained. This may represent the modified tyrosyl residues in the active center. It should be pointed out that due to experimental errors and incomplete protection, the above numbers must be considered as tentative figures. The better protecting agent oxaloacetate was not used because it absorbs ultraviolet light and is unstable.

TABLE I

EFFECT OF ACETYLATION ON THE APPARENT MICHAELIS CONSTANTS OF MALIC ENZYME

Substrate	Native enzyme	SH-reactivated enzyme [a]	Acetylated enzyme [b]
Malate	40 μM [c]	40 μM	200 μM
Oxaloacetate	70 μM	69 μM	175 μM
Pyruvate	13 mM [c]	12 mM	12 mM
NADP [d]	1.4 μM	1.7 μM	1.3 μM
NADPH [d,e]	2.9 μM	2.9 μM	3.2 μM

[a] Fully active in oxidative decarboxylase as compared to native enzyme.
[b] Residual oxidative decarboxylase activity was 20—55%.
[c] From Change and Hsu [10].
[d] Determination of K_m for the nucleotides was performed with Gilford 250 spectrophotometer equipped with a 10-cm cuvette compartment. Cylindrical cuvettes with a total volume of 30-ml and 10-cm light path were used.
[e] Determined in the reverse reaction of oxidative dercarboxylation.

Kinetic properties of the acetylated enzyme

In order to assess the effect of chemical modification on substrate affinities, the apparent Michaelis constants for substrates were determined on native and modified enzyme as shown in Table I. Kinetic analysis indicated that the acetylation of tyrosyl groups caused an increase of K_m for malate and oxaloacetate, but no change for pyruvate and nucleotides was observed. These results suggested that the decrease in oxidative decarboxylase and oxaloacetate decarboxylase activities may be due to a decrease in the affinity between the acetylated enzyme and malate or oxaloacetate. The decrease in reductase activity, on the other hand, is probably due to lower catalytic efficiency. However, the possibility should be pointed out that modification could, in fact, abolish binding of pyruvate. The observation of an unchanged K_m would merely indicate the presence of some unmodified enzyme.

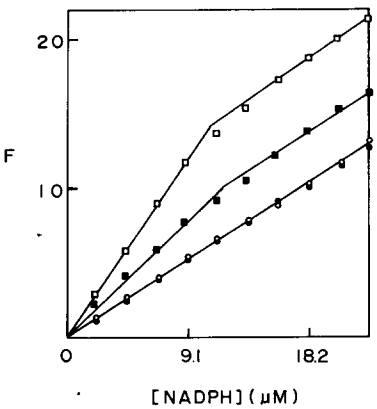


Fig. 7. Fluorescence titration of malic enzyme with NADPH. All cuvettes contained 50 mM Tris-HCl buffer (pH 7.0), 8.8 mM dithioerythreitol, 37.5 μM *p*-chloromercuribenzoate, 12.5 mM borate buffer (pH 7.5), and the followings: (○), no other component; (●), 25 mM *N*-acetylimidazole; (■), 1.27 μM acetylated enzyme with 8% residual activity; (□), 1.27 μM fully active SH-reactivated enzyme. Excitation was at 350 nm, emission at 470 nm.

To ascertain that acetylation did not abolish the nucleotide binding, the fluorescence titration experiment was performed. The fluorescence of NADPH was enhanced when the nucleotide was added into malic enzyme solution [6]. Fig. 7 shows the plot of corrected relative fluorescence value versus NADPH concentrations. Both SH-reactivated enzyme and acetylated enzyme gave fluorescence enhancement. Site dissociation constant was obtained graphically from the titration values shown in Fig. 7 near the equivalent point where both free enzyme and free NADPH concentrations were significant. The reciprocal plots of $1/F$ against 1/[NADPH] gave straight lines for the two enzyme preparations. The site dissociation constant for NADPH was found to be 18 and 33 μM for the SH-reactivated and acetylated enzyme, respectively. Thus NADPH binding was not abolished by acetylation. However, the affinity for NADPH appeared to be somewhat decreased.

Protection of malic enzyme from tetranitromethane modification by N-acetylimidazole

Additional proof that *N*-acetylimidazole and tetranitromethane reacted with the same tyrosyl residues of malic enzyme was obtained by a double modification experiment. SH-masked enzyme was incubated with *N*-acetylimidazole until 95% of the enzyme activity had lost. The acetylated enzyme was then incubated with tetranitromethane under the conditions that would destroy 99% enzyme activity. Hydroxylamine (1 M) was then added to the solution. 23% of the enzyme activity was recovered in 10 min as compared to the 77% without tetranitromethane treatment. The low recovery of the enzyme activity was probably due to irreversible cross-linking reaction, since *N*-acetylimidazole did not protect all tyrosyl residues.

Results of the present study provide evidences for the presence of functional tyrosine residues at the substrate binding site of pigeon liver malic enzyme. These results were similar to the acetylation of bovine heart malate dehydrogenase [32]. A tyrosine residue was also found to be essential for pig heart malate dehydrogenase [33]. At least when the dehydrogenase function was concerned, they might well share a common catalytic mechanism. As pointed out by Siegel and Ellison [32], the requirement of a compound to have two carboxyl groups to give protection suggested that some other amino acids may be involved in the substrate binding. We are now investigating this possibility.

Acknowledgements

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References

1 Ochoa, S.A., Mehler, A.H. and Kornberg, A. (1947) J. Biol. Chem. 167, 871—872
2 Ochoa, S.A., Mehler, A.H. and Kornberg, A. (1948) J. Biol. Chem. 175, 979—1000
3 Salles, J.B.V. and Ochoa, S. (1950) J. Biol. Chem. 187, 849—861
4 Hsu, R.Y. and Lardy, H.A. (1967) Acta Biochem. Pol. 14, 183—186
5 Tang, C.L. and Hsu, R.Y. (1973) Biochem. J. 135, 287—291
6 Hsu, R.Y. and Lardy, H.A. (1967) J. Biol. Chem. 242, 527—532
7 Nevaldine, B.H., Bassel, A.R. and Hsu, R.Y. (1974) Biochim. Biophys. Acta 336, 283—293
8 Tang, C.L. and Hsu, R.Y. (1974) J. Biol. Chem. 249, 3916—3922
9 Chang, G.G. and Hsu, R.Y. (1973) Biochem. Biophys. Res. Commun. 55, 580—587
10 Chang, G.G. and Hsu, R.Y. (1977) Biochemistry 16, 311—320
11 Chang, G.G. and Hsu, R.Y. (1977) Biochim. Biophys. Acta 483, 228—235
12 Pry, T. and Hsu, R.Y. (1978) Biochemistry 17, 4024—4029
13 Chang, G.G. and Huang, T.M. (1979) Biochem. Biophys. Res. Commun. 86, 829—836
14 Chang, G.G. (1979) J. Chin. Biochem. Soc. 8, 6p (Abstract)
15 Hsu, R.Y. and Lardy, H.A. (1967) J. Biol. Chem. 242, 520—526
16 Bergmeyer, H.M. (1965) Methods of Enzymatic Analysis, p. 953, Academic Press, New York
17 Kosicki, G.W. (1968) Biochemistry 7, 4299—4302
18 Beaven, G.H. and Gratzer, W.B. (1968) Biochim. Biophys. Acta 168, 456—462
19 Hsu, R.Y. (1970) J. Biol. Chem. 245, 6675—6682
20 Levy, H.M., Leber, P.D. and Ryan, E.M. (1963) J. Biol. Chem. 238, 3654—3659
21 Riordan, J.F., Sokolovsky, M. and Vallee, B.L. (1966) J. Am. Chem. Soc. 88, 4104—4105
22 Lindley, H. (1962) Biochem. J. 82, 418—425
23 Cohn, E.J. and Edsall, J.T. (1943) Protein, Amino Acids, and Peptides as Ions and Dipolar Ions, Reinhold, New York
24 Sokolovsky, M., Riordan, J.F. and Vallee, B.L. (1966) Biochemistry 5, 3582—3589
25 Tsukamoto, S. and Ohno, M. (1978) J. Biochem. 84, 1625—1632
26 Doyle, R.J., Bello, J. and Roholt, O.A. (1968) Biochim. Biophys. Acta 160, 274—276
27 Vincent, J.P., Lazdunski, M. and Delaage, M. (1970) Eur. J. Biochem. 12, 250—257
28 Kivschner, M.W. and Schachman, H.K. (1973) Biochemistry 12, 2987—2997
29 Bunning, P., Holmquist, B. and Riordan, J.F. (1978) Biochem. Biophys. Res. Commun. 83, 1442—1449
30 Means, G.E. and Feeney, R.E. (1971) Chemical Modification of Proteins, pp. 72—74, Holden-Day, San Francisco, CA
31 Hsu, R.Y., Lardy, H.A. and Cleland, W.W. (1967) J. Biol. Chem. 242, 5315—5322
32 Siegel, L. and Ellison, J.S. (1971) Biochemistry 10, 2856—2862
33 Otwell, H.B., Tan, A.Y-H. and Friedman, M.E. (1978) Biochim. Biophys. Acta 527, 309—318