FUNCTIONAL ARGININE RESIDUES INVOLVED IN COENZYME BINDING BY DIHYDROFOLATE REDUCTASE

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SUMMARY

Reaction of dihydrofolate reductase from amethopterin-resistant Lactobacillus casei with phenylglyoxal results in a complete loss of enzyme activity. This inactivation is concomitant with the modification of five of a total of eight arginine residues per mole of enzyme. In the presence of the reduced coenzyme, NADPH, two of the five reactive arginines are protected from chemical modification with complete retention of enzyme activity. The results suggest the involvement of essential arginine residues at or near the coenzyme binding site and thus at or near the active center of the enzyme.

INTRODUCTION

Arginine residues appear to be involved in coenzyme binding in a number of pyridine nucleotide-dependent dehydrogenases (1-5). Yang and Schwert (1) found that modification of arginine residues of bovine H4 lactate dehydrogenase by butanedione resulted in enzyme inactivation. X-ray crystallographic studies of the dogfish M4 lactate dehydrogenase-NAD complex by Adams et al. (2) have shown that arginine-101 moves ca. 13 A to interact with the pyrophosphate bridge of the coenzyme. In addition, arginine residues have been implicated in coenzyme binding in alcohol dehydrogenases (3), glutamate dehydrogenases (4) as well as in malate dehydrogenases (5).

The reduced coenzyme, NADPH, is required by dihydrofolate reductase (E.C.1.5.1.3) for the reduction of L-7,8 dihydrofolate to 1,L-5,6,7,8-tetrahydrofolate, the coenzyme carrier involved in a variety of one-carbon

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transfer reactions. In the present communication the possible functional role of arginine residues in coenzyme binding by dihydrofolate reductase is evaluated by means of chemical modification with phenylglyoxal.

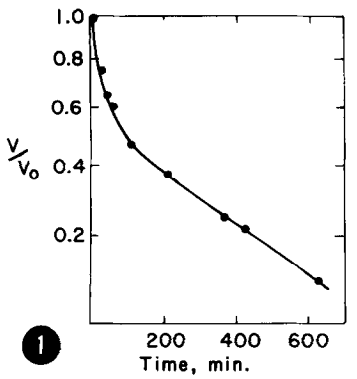
MATERIALS AND METHODS

Dihydrofolate reductase from an amethopterin-resistant strain of Lactobacillus casei was isolated and purified by a modification (6) of the procedure of Gunderson, et al. (7). Electrophoretic form I, which does not contain bound NADPH, was employed in these studies. Enzyme assays were performed spectrophotometrically at 25° by following the decrease in absorbance at 340 nm using a Gilford Model 240 spectrophotometer and a Sargent Model SRLG recorder. The assay mixture (1.2 ml) contained 60 mmoles KPO_4 buffer, pH 7.0, 50 µmoles of NADPH (P-L Biochemicals), 40 µmoles of dihydrofolate and enzyme (ca. 1×10^{-7} M). Dihydrofolate was prepared by the reduction of folic acid (Cyclo chemicals) with sodium dithionite according to the method of Blakley (8). Enzyme modifications with phenylglyoxal (K and K Labs) were done at 20° in 0.1 M KPO_4, pH 7.8. Amino acid analyses were performed in a Durrum D-500 amino acid analyzer following hydrolysis in vacuo in 6 N HCl for 20 hr at 110° according to the general procedures of Moore and Stein (9).

RESULTS AND DISCUSSION

Solutions of dihydrofolate reductase (5×10^{-5} M) were incubated with a 200-fold molar excess of phenylglyoxal. Control samples were incubated under identical conditions without the addition of reagent. The effect of phenylglyoxal on the enzymatic activity of dihydrofolate reductase is shown in Figure 1. The fractional activity remaining is expressed as the log of the ratio V/Vo, where V is the enzyme activity of the phenylglyoxal-modified reductase and Vo is the activity of an unreacted control.

Aliquots of the enzyme-phenylglyoxal reaction mixture were removed at various intervals during the time course of the inactivation. The reaction at each interval was terminated by the addition of 6 N HCl and, after acid hydrolysis of the precipitated protein, amino acid analysis was performed on each sample. As indicated in Figure 2, the complete inactivation of dihydrofolate reductase corresponds to a loss of five of a total of eight arginine residues per mole of protein. Comparison of the analyses for the native and inactivated enzymes indicate that no amino acid other than arginine is modified. Many of the highly polar guanidino groups of arginine residues would, presum-



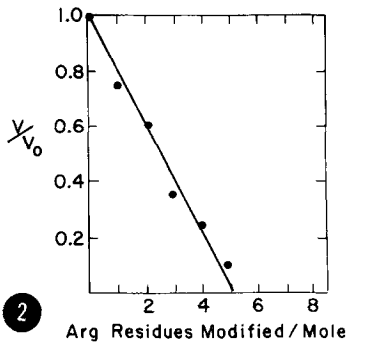


FIGURE 1. Inactivation of dihydrofolate reductase by phenylglyoxal. Enzyme (2×10^{-5} M) in 0.1 M KPO_4, pH 7.8, was incubated with 4×10^{-3} M phenylglyoxal at 20°. Aliquots were removed at the indicated times for assay.

FIGURE 2. Decrease of dihydrofolate reductase activity as a function of arginine modification. Enzyme (5×10^{-5} M) was reacted with a 200-fold molar excess of phenylglyoxal as described in Figure 1. Aliquots of the inhibition mixture were treated as described in the text and amino acid analyses performed to determine the residues of arginine present.

ably, be exposed on the surface of the protein. In the present work more than half of the total arginines are modified by phenylglyoxal.

In an attempt to distinguish essential from non-essential arginine residues, dihydrofolate reductase was pre-treated with reduced pyridine nucleotide prior to chemical modification. Incubation of the reductase with NADPH prior to treatment with phenylglyoxal results in complete protection against enzyme inactivation. However, approximately three arginine residues are modified without loss in enzymatic activity as shown in Figure 3. Following removal of the reagent and reduced coenzyme, the reductase is inactivated in the presence of phenylglyoxal. Of the five reactive arginine residues in dihydrofolate reductase, two such residues are rendered inaccessible in the presence of NADPH, suggesting that these residues are essential to coenzyme binding and are, therefore, at or near the active center of the enzyme.

Recently, Feeney et al. (10) have demonstrated that the 2'-phosphate group of either NADPH or $NADP^+$ is in the dianionic state in binding complexes

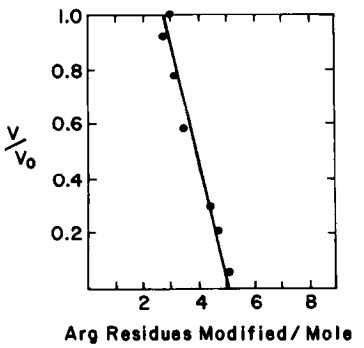


FIGURE 3. Effect of phenylglyoxal on the activity of dihydrofolate reductase in the presence and absence of NADPH. Enzyme (5×10^{-5}M) was incubated for five minutes with 5×10^{-4}M NADPH in 0.1M KPO_4, pH 7.8, prior to the addition of a 200-fold molar excess of phenylglyoxal to enzyme. Following reaction for two hours at 20° the reaction mixture was passed through a 2.5 x 45 cm Sephadex G-25 column equilibrated with pH 7.8 phosphate buffer to remove the reagent and NADPH. The enzyme was again treated with phenylglyoxal as described above; enzyme activity and arginine contents were determined as described in Figures 1 and 2, respectively.

with L. casei dihydrofolate reductase, based on ^{31}P NMR spectral data. The chemical shift and position of the 2'-phosphate resonance in either dinucleotide enzyme complex is independent of pH in the range of 4.5 to 7.5, indicating that the pK of the 2'-phosphate group differs by at least three units from that found for either of the free coenzymes alone in solution. Such a drastic alteration in the pK of the 2'-phosphate group would strongly support the possibility of an electrostatic interaction between this moiety and one or more cationic groups on the protein. The permanent positive charge of the guanidino moiety of one or more arginine residues would form a strong salt bridge with a dianionic phosphoryl group and, therefore, make a significant contribution in terms of the energy of dinucleotide binding. In addition, electrostatic interactions between the pyrophosphate group of NADPH and arginine residues may play a role in pyridine nucleotide binding by dihydrofolate reductase. Such interactions have been shown to be important in the binding of NAD^+ to dehydrogenases as was previously indicated.

Studies are now in progress to determine the location of these arginine

residues in the amino acid sequence of dihydrofolate reductase. The unique localization of one or more arginine residues near the coenzyme 2'-phosphate in the NADPH binding domain may well provide, at least in part, the structural basis for phosphorylated dinucleotide binding by dihydrofolate reductase

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