THIOLYSIS OF S-DINITROPHENYLATED CREATINE KINASE WITH RESTORATION OF ENZYMATIC ACTIVITY

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SUMMARY: Creatine kinase has been shown to be stoichiometrically inhibited by 1-fluoro-2,4-dinitrobenzene which reacts with the reactive sulfhydryl group on each polypeptide chain of the enzyme. Enzymatic activity is restored by thiolysis of S-dinitrophenylated creatine kinase with a 12-fold molar excess of 2-mercaptoethanol at pH 9 and 30°C. The S-dinitrophenylated creatine kinase is stable for two hours at pH 7 and 30°C as measured by the ability of 2-mercaptoethanol to restore enzymatic activity but after one hour at pH 9 and 30°C only 50% of the enzymatic activity can be restored.

Creatine kinase (adenosine 5'-triphosphate-creatine transphosphorylase EC 2.7.3.2) from rabbit muscle reacts stoichiometrically with 1-fluoro-2,4-dinitrobenzene (FDNB) to form a S-dinitrophenylated creatine kinase derivative.[1] Shaltiel demonstrated the quantitative removal of the dinitrophenyl (DNP) group from several dinitrophenylated side chain groups of amino acids by thiolytic cleavage with 2-mercaptoethanol.[2] This paper describes the application of thiolysis to remove the DNP group from S-DNP-creatine kinase with almost full restoration of enzymatic activity.

MATERIALS AND METHODS

Creatine kinase was prepared from rabbit muscle as previously described,

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and had a specific activity of 60-72 units/mg.[1,3,4] The enzyme concentration was determined by the biuret method and by absorption at 280 mμ.[4,5] 1-Fluoro-2,4-dinitrobenzene (FDNB) was purchased from Pierce Chemical Company and 2-mercaptoethanol from Eastman. To maintain a constant temperature and pH in these studies a Radiometer TTT-1 pH meter, SBU titrograph, and SBU-1 syringe assembly were used with the sample contained in a jacketed reaction vessel.

Effect of pH on Restoration of Enzymatic Activity by 2-Mercaptoethanol: A solution of creatine kinase (34.5 mg/ml) containing 2.5 μmoles of reactive sulfhydryl groups (based on a molecular weight of 41,300[6] for each polypeptide chain) at 0°C was adjusted to the required pH with 0.01 N HCl or 0.05 N NaOH. An equivalent amount (2.5 μmoles) of FDNB in isopropyl alcohol was added with mixing. After approximately 5 minutes at 0°C a 12-fold molar excess of 2-mercaptoethanol in isopropyl alcohol was added. The temperature of the solution was raised to 30°C and the pH readjusted to that required. Samples for the determination of enzymatic activity were removed at 5-minute intervals after the addition of 2-mercaptoethanol. The results obtained at pH 7.0, 8.0, and 9.0 are shown in Figure 1.

Identification of S-DNP-2-Mercaptoethanol as the Thiolysis Product of S-DNP-Creatine Kinase: The samples of S-DNP-creatine kinase treated with 2-mercaptoethanol described above were pooled and extracted several times with ether. The combined ether extracts were evaporated and chromatographed on Whatman #1 paper in isoamyl-pyridine-water (35:35:30) system.[2] An authentic sample of S-DNP-2-mercaptoethanol was prepared[7] and similarly chromatographed. Only one yellow spot with R_f 0.88 was observed for each sample. Both samples, when eluted from the chromatogram with ethanol, had the same ultraviolet absorption spectrum comparable to that reported for S-DNP-2-mercaptoethanol.[2]

Effect of pH on Stability of S-DNP-Creatine Kinase: A solution of creatine kinase (41.0 mg/ml) containing 10 μmoles of reactive sulfhydryl groups was inactivated at pH 7 or 9 and 0°C with an equimolar amount of FDNB in isopropyl

alcohol. A sample was removed at this time to test for completeness of inactivation. The solution was maintained at the required pH with 0.01 N HCl or 0.05 N NaOH. At various intervals, including zero time, 2-ml samples were removed and assayed for enzymatic activity after incubation with a 12-fold molar excess of 2-mercaptoethanol at pH 9 and 30°C for 30 minutes. In calculating per cent restoration of enzymatic activity the change in concentration due to addition of reagents was taken into consideration. The results obtained at pH 7 and 9 are shown in Figure 2. At pH 9 the intensity of the yellow color of the solution increased noticeably with time.

RESULTS AND DISCUSSION

Figure 1 shows the effect of incubating S-DNP-creatine kinase at 30°C with a 12-fold molar excess of 2-mercaptoethanol at various pH concentrations. At pH 9 there is almost complete restoration of enzymatic activity within 30 minutes. These results offer additional evidence that the reactive sulfhydryl groups in creatine kinase are at the active sites of the enzyme.

S-DNP-2-Mercaptoethanol was identified as the cleavage product confirming the thiolysis mechanism proposed by Shaltiel.[2]

The fact that S-DNP-creatine kinase can be deblocked to give enzymatically active creatine kinase led us to investigate the stability of the enzyme derivative at various pH concentrations. Figure 2 shows the results of this study which demonstrate the stability of the enzyme derivative at pH 7 and 30°C even after two hours, indicating that at least a non-reversible conformational change does not occur when the DNP group is attached to the enzyme. Such a conformational change was a possible explanation for the loss of enzymatic activity upon reaction with FDNB.[1] However at pH 9 and 30°C about half of the ability of 2-mercaptoethanol to reactivate S-DNP-creatine kinase is rapidly lost and then the rate of change decreases. It was also observed that the yellow color of the solution becomes more intense indicating that there may be a migration of the DNP group to another

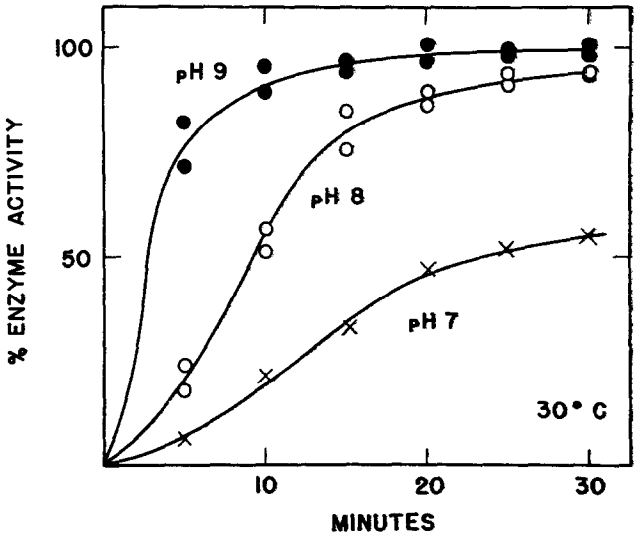

Figure 1. The effect of pH on the ability of 2-mercaptoethanol to remove the DNP group from S-DNP-creatine kinase and restore enzymatic activity. On the ordinate is plotted the per cent of the original enzymatic activity. At zero time a 12-fold molar excess of 2-mercaptoethanol was added to 3 ml of creatine kinase at 0°C. The temperature was then raised to 30°C. Samples were removed for enzymatic assay at the time of the addition of the 2-mercaptoethanol and for 30 minutes at 5 minute intervals.

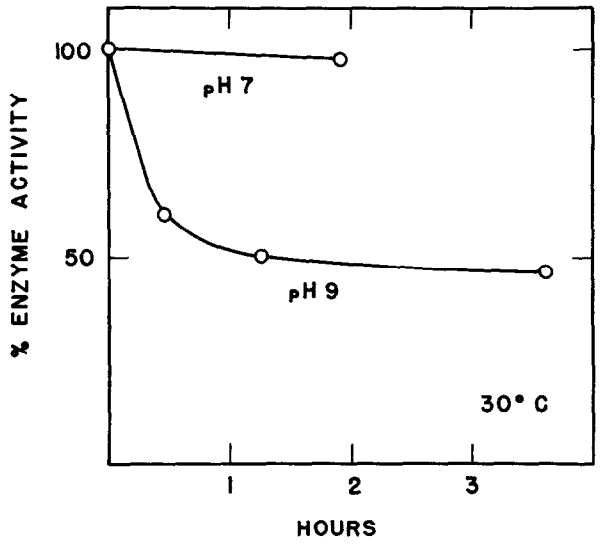

Figure 2. The effect of pH on the stability of S-DNP-creatine kinase at 30°C. On the ordinate is plotted the per cent of the original enzyme activity. The zero time on the graph represents an initial sample treated with a 12-fold molar excess of 2-mercaptoethanol for 30 minutes at pH 9 and 30°C, followed by assaying the mixture for enzymatic activity. Additional samples were taken at the time intervals indicated on the graph and treated and assayed as was the initial sample.

position in the enzyme or that there may be a β-elimination[8] of thiophenol resulting in a dehydroalanine residue replacing the cysteine residue in the enzyme. The native enzyme itself is stable under these conditions. We are presently investigating the apparent non-reversible transformation of the DNP group under these conditions.

These results show that the reactive sulfhydryl groups in creatine kinase may be protected by dinitrophenylation to allow chemical modification of other functional groups in the enzyme. Subsequent thiolysis with 2-mercaptoethanol would result in a modified enzyme with the reactive sulfhydryl groups again exposed.

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