REVERSIBLE INACTIVATION OF AZOTOBACTER VINELANDII TPN^+ - ISOCITRATE DEHYDROGENASE BY THE FORMATION OF AN INTRAMOLECULAR DISULFIDE BRIDGE *

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SUMMARY

A method has been described for the formation of a modified form of isocitrate dehydrogenase which contains a single disulfide bridge. The modified enzyme is inactive and exhibits a decrease in α-helix content. The modified enzyme is inactive but catalytic activity can be restored by cleavage of the disulfide bridge. The original α-helix content is not restored upon reduction.

TPN^+ - Isocitrate dehydrogenase (IDH) from Azotobacter vinelandii contains 3 sulfhydryl groups per mole of enzyme (Chung and Franzen, 1969). One of the three sulfhydryl groups is unusually reactive and has been implicated in the active site of the enzyme. In this communication we wish to report a method for the preparation of a modified form of isocitrate dehydrogenase which contains an intramolecular disulfide bond. One of the sulfur atoms of the disulfide bond is donated by the reactive sulfhydryl group of the enzyme. The disulfide enzyme is inactive and can be partially reactivated by reduction with dithiothreitol (DTT). The conformational changes in the protein molecule which accompany the formation of the disulfide bridge and its subsequent reduction offer an unusual opportunity to correlate changes in catalytic activity with the tertiary structure of the protein. It may be possible to apply the method described here for the

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formation of an intramolecular disulfide bridge to other sulfhydryl containing enzymes.

EXPERIMENTAL AND RESULTS

Isocitrate dehydrogenase (IDH) was prepared as previously described (Chung and Franzen, 1969). The disulfide monomer was prepared from IDH in the following manner. IDH, 90 mg, was dissolved in 44 ml of 0.05 M potassium phosphate buffer (KPO_4) pH 7.0 and mixed witn 0.5 ml of a solution of 5,5'-dithiobis (2-nitro benzoic acid) (DTNB) at a concentration of 4 mg/ml of 0.05 M KPO_4, pH 7.0 (Ellman, 1959). The solution was allowed to stand in the dark at room temperature. The course of the reaction was monitored by measuring the absorbance of the solution at 412 nm periodically. The reaction was complete within about 30 minutes at which time approximately one mole of thiol per mole of enzyme was titrated. This enzyme preparation contained less than 5% of the activity of the native enzyme. Complete recovery of catalytic activity could be obtained by the addition of excess DTT. The solution containing the titrated enzyme (TNB-IDH) was filtered through a Sephadex G-25 column (6 x 50 cm) to remove excess DTNB and released TNB chromophore. The protein containing solution which was eluted from the column with 0.05 M KPO_4 pH 7.0 was collected and mixed with guanidine HCl solution to yield a final concentration of guanidine HCl of 0.5 M. The final volume was 200 ml and the protein concentration 0.42 mg/ml. The almost colorless solution was allowed to stand in ice for 120 minutes. At the end of this time the solution became yellow and measurement of its absorbance at 412 nm indicated that approximately 1 equivalent of TNB was released per mole of protein. The released chromophore and guanidine HCl were removed from this solution by gel filtration through Sephadex G-25. The resulting protein solution in 0.05 M KPO_4 pH 7.0 was concentrated by ultrafiltration with an Amicon ultrafilter cell (Amicon Corporation, Lexington, Mass.) to a volume of

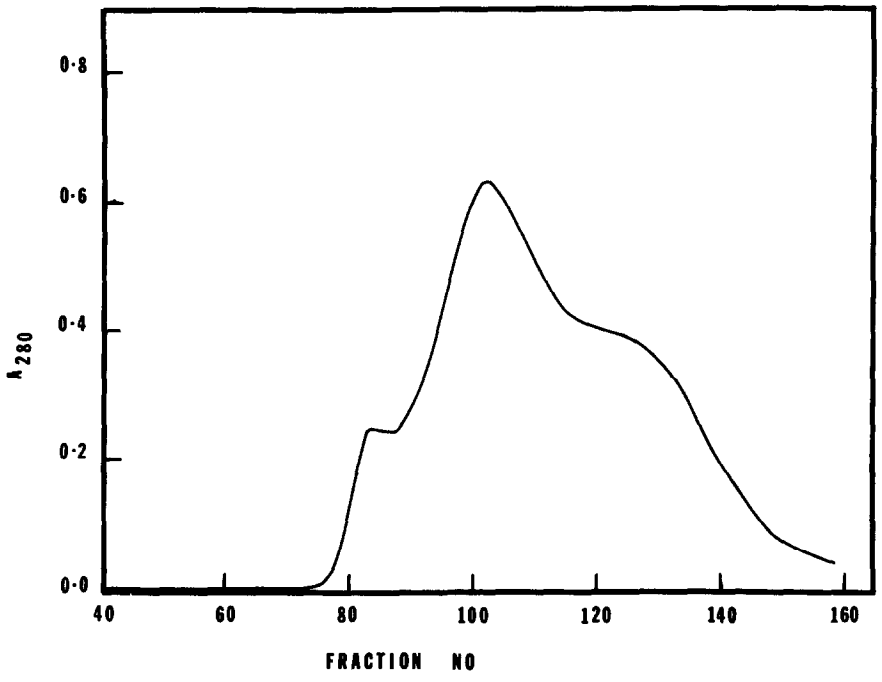

Figure 1 : Elution pattern of protein from Sephadex-G-150.

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13 ml. 6.5 ml of this solution which had a total of 36 mg of protein were chromatographed on a Sephadex G-150 column (2.5 x 90 cm) equilibrated with 0.05 M KPO_4 pH 7.0. Fractions of approximately 3 ml were collected at a flow rate of 40 ml/hour. The elution pattern for protein is shown in Figure 1. Fractions 110 to 135 were pooled and concentrated by ultrafiltration to yield 4.5 ml of solution containing 4.6 mg protein/ml.

The enzyme preparation thus obtained was analyzed in the ultracentrifuge. The sedimentation pattern indicated that the preparation was homogeneous and had an $s^{\circ}_{20,w}$ of 4.6×10^{-13} sec. This sedimentation constant is identical to that of the native enzyme.

The sulfhydryl content of the modified enzyme was determined by titration with p-hydroxymercuribenzoate (PMB) by the method of Boyer (1954). In Table I the sulfhydryl contents of several forms of IDH are compared. The modified enzyme species is referred to as disulfide monomer.

Table I. Sulfhydryl Content of Different Forms of IDH

Enzyme Species	Moles - SH/Mole Enzyme
IDH	3.32
TNB-IDH	1.97
Disulfide Monomer	1.02
Reactivated Monomer	3.05

Titration was carried out according to the method of Boyer (1954). The molar concentration of enzyme was calculated from the absorbancy of the enzyme solution at 280 nm using a value $A_{280}^{1\%}$ of 8.9 and assuming a molecular weight of 80,000 (Chung and Franzen, 1969).

It may be seen that the native enzyme contains 3 -SH groups/80,000 g of protein, TNB-IDH contains 2 -SH groups/mole enzyme and modified enzyme (disulfide monomer) contains only 1 -SH group/mole enzyme. After reduction of the disulfide monomer with 0.01 M DTT and removal of excess DTT by gel filtration, the resulting enzyme, referred to as reactivated monomer, contained 3 -SH groups/mole enzyme.

The disulfide monomer was essentially devoid of catalytic activity. Reduction of the disulfide bond with DTT resulted in a rapid regeneration of catalytic activity. Maximal regeneration of catalytic activity was obtained by incubation of the disulfide monomer with 5×10^{-3} M DTT for 12-24 hours at 0° in 0.05 M KPO_4 pH 7.0. The specific activity of the regenerated enzyme varied between 50 and 90 enzyme units/mg protein. The native enzyme has a specific activity of 130 enzyme units/mg protein.

The circular dichroism spectra of the native IDH, disulfide monomer, and regenerated monomer are shown in Figure 2. The native enzyme has an α-helix content of approximately 30% calculated from the $[\theta']$ values at 193 and 222 nm (Chung and Franzen, 1969). Similar calculations for the disulfide monomer indicate that this species contains approximately 30%

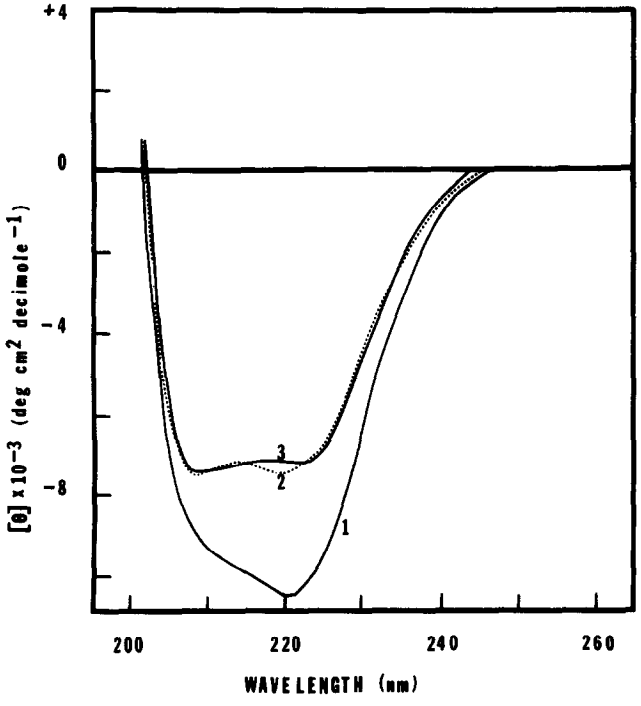
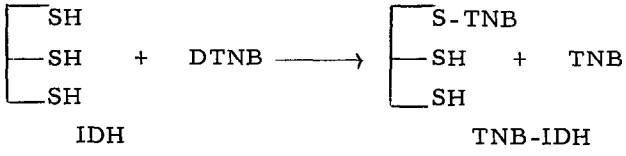


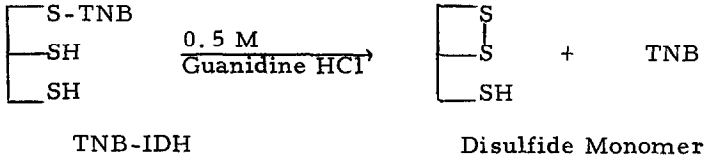
Figure 2: Circular dichroism spectra of different forms of IDH. Curve 1, native IDH; curve 2, disulfide monomer; curve 3, reactivated monomer. Circular dichroism spectra were obtained with a Cary 6002 recording spectropolarimeter. The samples were maintained at 25° and the solvent was 0.05 M KPO_4 pH 7.0. The specific activities of the native enzyme, disulfide monomer and regenerated enzyme were 130, < 5 and 60 enzyme units/mg protein respectively.

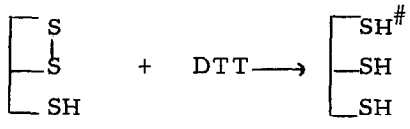
less α-helix than the native enzyme. Reduction of the disulfide bond does not appear to restore the native enzyme conformation although activity is regenerated. It is clear, however, that the introduction of a single disulfide bond into the enzyme molecule fixes it into a new and inactive conformation. The reactivated enzyme contained 3 -SH groups/mole enzyme but its specific activity was only 60 enzyme units/mg protein. Since it did not assume the conformation of the native enzyme it is possible that a new stable conformation of the enzyme with decreased specific activity was obtained or alternatively the reduced enzyme contained a mixture of conformers with varying specific activities. It is not known at the present time which alternative is correct.

DISCUSSION

The results obtained above are schematically summarized in equations (1) to (3).

(1) 

(2) 

(3) 

The results obtained are of interest from at least two points of view. Firstly, the ready formation of the disulfide enzyme and its reactivation may serve as a model to correlate conformational changes with catalytic activity. It might be noted that the active center thiol is protected and therefore further modification of the enzyme with reporter groups is feasible. The subsequent reduction of the disulfide bridge and the conformational changes which accompany the regeneration of catalytic activity might then be monitored. Furthermore the third thiol residue which is normally buried within the molecule becomes accessible to attack by a variety of thiol reagents in the disulfide enzyme. By labeling this group with an appropriate reagent, changes in its environment upon reactivation of the enzyme molecule may be studied. It may be possible to extend these results to other sulfhydryl enzymes. Indeed, Boross (1969) has observed that the sulfhydryl groups of D-glyceraldehyde-3-phosphate dehydrogenase (GADP) exhibit somewhat similar properties to those of IDH described here. The TNB-group in TNB-GADP is spontaneously expelled by incubation at room temperature with the formation of intramolec-

ular disulfide bridges. The second area of interest in these observations is the possible role of the reversible formation of disulfide bridges in the regulation of catalytic activity in vivo. It may be construed that the reversible formation of disulfide bridges could serve several functions viz (a) reversible inactivation of the enzyme according to the physiological needs of the cell, (b) protection of a sensitive thiol group, (c) provision of a pathway for the interconversion of different conformational states of the enzyme with different activities.

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