UCRL-12192

JAN 4 1964

MASTER

University of California

Ernest O. Lawrence Radiation Laboratory

This paper was submitted for publication in the open literature at least 18 months prior to the issuance date of this Microcard. Since the U.S.A.E.C. has no evidence that it has been published, the paper is being distributed in Microcard form as a preprint.

CONFORMATION CHANGES IN CITRATE-CONDENSING ENZYME

RELEASED FOR ARCHIVEMENT

IN NUCLEAR SCIENCE ABSTRACTS

Livermore, California

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

UNIVERSITY OF CALIFORNIA
Lawrence Radiation Laboratory
Livermore, California

Contract No. W-7405-eng-48

CONFORMATION CHANGES IN CITRATE-CONDENSING ENZYME

Paul A. Srere

November 11, 1964

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

Summary

The four sulfhydryl groups of citrate-condensing enzyme will not react in aqueous solution with 5,5'-dithiobis(2-nitrobenzoate) (DTNB). When urea is added to the enzyme the conformation of the protein is changed in a way that permits reaction between DTNB and protein SH. The rate of "unfolding" is first-order and dependent on urea concentration and temperature. The conformation change induced by urea can be slowed by the addition of substrates.

A number of physical and chemical methods are available to study the changes in conformation of proteins (1-3). One of these methods is based on the observation that the sulfhydryl groups (SH) of proteins vary in their ability to react with SH reagents depending upon their location in the tertiary structure of the protein (4-6). In some proteins the SH groups react immediately with SH reagents while in others the SH groups react only after changes have occurred in the tertiary structure of the protein, i. e., those "denaturations" that occur in high molar concentrations of urea or in the presence of various surface-active compounds.

A wide range of SH reagents is available to study the availability of SH groups on proteins but the best of these is 5,5'-dithiobis(2-nitrobenzoate) (DTNB), introduced by Ellman (7). One product of the reaction of RSH and DTNB is the mercaptide of nitrobenzoate which has an absorption maximum at about 412 $m\mu$ and a molar extinction coefficient of 13,600.

Ellman has shown that while the SH of blood proteins reacts slowly with DTNB, the reaction is rapid when carried out in 50% acetone or 8 M urea; this indicates that under the conditions usually employed the protein SH groups are in an unfavorable environment (either from electronic or physical configuration) for this reaction. Recently Bitensky et al. (8,9) have used DTNB to follow the "uncovering" of SH groups that occurs during dissociative changes of sub-units in glutamate dehydrogenase, and have also observed SH release when additional conformation changes occur in the sub-units. We have observed recently that the four SH groups of citrate-condensing enzyme are available to reaction with DTNB only after conformational changes in the protein (10). The present paper reports some of the kinetic properties of the reactions of the SH groups of citrate-condensing enzyme which are exposed during changes in protein conformation.

Materials and Methods

Crystalline citrate-condensing enzyme prepared from pig heart (11) was assayed by following CoASH production with DTNB as previously described (12). The DTNB was purchased from Aldrich; the urea was Baker's Reagent Grade. No urea solution was more than one week old; however, no difference in the SH content or the rate of reaction was observed when a two-week-old and a freshly prepared solution were compared. Commercial Duponol was recrystallized twice from ethanol. Reactions were followed using the 0-0.200 absorbancy scale of a Gilford attachment to a Beckman monochromator equipped with thermospacers.

Results

The SH groups of the enzyme do not react with DTNB in aqueous solution but do so in urea (Fig. 1). The results in Fig. 1 also show that DTNB does not affect the unfolding of the protein, since after 6 min all the SH groups are available either with or without DTNB. The reaction between the SH groups of citrate-condensing enzyme and DTNB is first-order for over 90% of the reaction (Fig. 2). Using an estimate of 80,000 for the molecular weight one can calculate four moles of SH per mole of enzyme. The apparent first-order rate constant is not altered either by a tenfold change in enzyme concentration (from 10^{-6} M to 10^{-5} M) (Fig. 2) or by a tenfold change in the concentration of DTNB (10^{-4} M to 10^{-3} M).

The rate of SH release was markedly affected by changes in urea concentration (Fig. 3). A plot of the log of k' , the apparent first-order rate constant, versus the log of the urea concentration yields a line of slope 3.7 (inset of Fig. 3).

The difference spectrum of enzyme in water and enzyme in urea (Fig. 4) is typical of results reported for other proteins. The first-order rate constant for the change in absorption at 260 $m\mu$ is about one-third that for the appearance of SH groups. Duponol at a concentration of 0.005% also causes a rapid appearance of SH from the enzyme.

The effect of temperature on the process in urea is shown in Fig. 5. An activation energy of 22 K cal can be calculated from the slope of the line.

When the enzyme is placed in urea it loses its activity. It can be seen in Fig. 6 that the rate constants for loss of activity in urea and for appearance of SH follow the same kinetics. However, the loss of enzyme activity is independent of the presence or absence of DTNB (Fig. 7). Since the urea concentration is reduced at least 100-fold when the enzyme is diluted for assay, the inactivation must be due to irreversible denaturation. We have found that either acetyl CoA, oxalacetate, or citrate will prevent both the appearance of SH from enzyme in urea and the loss of activity in urea (Table I), so that it is not possible to test for a reversible denaturation step by assaying enzyme activity in urea.

DISCUSSION

Many proteins contain SH groups that are unreactive with DTNB: besides the citrate-condensing enzyme reported in this paper there are the blood proteins reported by Ellman (7), glutamate dehydrogenase (8,9), β -lactoglobulin (13), and ovalbumin (13). In addition Rabin and Trown (14) have reported that SH groups of carboxydismutase exhibit an initial fast reaction followed by a slow reaction. Schramm has shown that the SH groups of α -amylase react with DTNB only after removal of metals and treatment with sodium dodecyl sulfate (15). Several reasons may be advanced to explain the

lack of rapid reaction between protein SH and DTNB. It is conceivable that the SH groups are buried in the interior of the protein thus making it difficult for the rather large molecule of DTNB to approach them. Perhaps the electronic environment of the SH groups also hinders the approach of the negatively charged DTNB molecule. One would imagine that the reactivity of protein SH groups with various SH reagents would give information as to the tertiary structure of the protein. Thus the SH groups of citrate-condensing enzyme react easily and rapidly with low concentrations of Hg^{++} with subsequent protein precipitation, they react partially with p-chlormercuribenzoate with subsequent precipitation, and they react not at all with N-ethylmaleimide or iodoacetamide. Although the SH groups are not involved in the active center, their position seems to be important, since a change resulting in altered reactivity with DTNB leads to loss of activity. It is possible, as suggested by Boyer, that these "buried" SH's are hydrogen-bonded to maintain the tertiary structure of the protein. It is interesting to note that a plot of the log of k' against the log of urea concentration gives a straight line with a slope of 3.7. This may be interpreted as meaning that 4 moles of urea are involved in the reaction of 1 mole of enzyme or that 1 mole of urea is needed to expose 1 mole of SH. These results do not define the mechanism of action of urea as the breakage of hydrogen bonds; additional information is required concerning the effects of other denaturing compounds (16).

DTNB offers several advantages in the study of changes in protein conformation. First, the colored product of the reaction is the same for all SH groups, hence the absorbancy index for the reaction is the same for all SH proteins. Second, absorbancy is high and occurs at $412 \text{ m}\mu$, where most proteins have little or no absorption. Lastly, it seems to be very selective with respect to the environment required for reaction with the SH groups. With

a protein such as the condensing enzyme, which has only four moles of SH per mole of protein, it is hoped that this reaction will yield information concerning the tertiary structure of the protein and the mechanism of denaturation.

References

1. Scheraga, H. A., "Protein Structure." Academic Press, New York, 1961.
2. Reithel, F. J., Advan. Protein Chem. 18, 124 (1963).
3. Schellman, J. A., and Schellman, C., in "The Proteins," Vol. II (H. Neurath, ed.) 2nd ed., p. 1. Academic Press, New York, 1964.
4. Boyer, P. D., in "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrback, eds.), p. 511. Academic Press, New York, 1959.
5. Boyer, P. D., Brookhaven Symposia Biol. 13, 1 (1960).
6. Cecil, R., in "The Proteins," Vol. I (H. Neurath, ed.), 2nd ed., p. 380. Academic Press, New York (1963).
7. Ellman, G., Arch. Biochem. Biophys. 82, 70 (1959).
8. Bitensky, M. W., Yielding, K. L., and Tomkins, G. M., Vith Intern. Cong. Biochem. Abstracts IV, 298 (1964).
9. Bitensky, M. W., Yielding, K. L., and Tomkins, G. M., J. Biol. Chem., in press.
10. Srere, P. A., Biochem. Biophys. Research Comm., in press.
11. Srere, P. A., and Kosicki, G. W., J. Biol. Chem., 236, 2559 (1961).
12. Srere, P. A., Brazil, H., and Gonen, L., Acta Chem. Scand., 17, S129 (1963).
13. Fernandez Diez, M. J., Osuga, O. T., and Feeney, R. E., Arch. Biochem. Biophys., 107, 449 (1964).
14. Rabin, B. R., and Trown, P. W., Nature 202, 1290 (1964).
15. Schramm, M., Biochemistry, 3, 1231 (1964).
16. Gordon, J. A., and Jencks, W. P., Biochemistry, 2, 47 (1963).

TABLE I

Effect of Substrates on Urea-Induced SH Release

Additions	% SH in 10 min
None	83
Oxalacetate, 20 μ moles	<5
Citrate, 100 μ moles	24
Acetyl CoA, 0.5 μ moles	50

Each cuvette contained 100 μ moles Tris-HCl pH 8.1, 100 μ moles DTNB, 100 μ g enzyme in a total volume of 1.0 ml 3.2 M urea.

GLL-6411-3288

Fig. 1. SH appearance in urea. Each cuvette contained finally 100 μ moles Tris-HCl pH 8.1, 1.3 μ moles enzyme (100 μ g), 100 μ moles DTNB, and the indicated urea concentration in a final volume of 1.0 ml. For the curves labeled 0.0 M urea, 4 M urea, and 7.2 M urea, the last addition to the cuvette was enzyme, and at that point the absorbancy at 412 $m\mu$ was recorded for the time indicated. For the curve labeled 4 M urea DTNB 6 min, the enzyme was incubated in the urea for 6 min, then DTNB was added and the absorbancy was recorded.

GLL-6411-3287

Fig. 2. Effect of enzyme concentration on rate of SH appearance. Each cuvette contained 100 μ moles Tris-HCl pH 8.1, 100 μ moles DTNB, urea to give a final concentration of 4 M, and either 100 μ g or 1 mg of enzyme as specified, in a final volume of 1.0 ml. Absorbancy at 412 $m\mu$ was recorded and the log of (total-SH at 30 min)/(SH remaining at time t) is plotted against time. The temperature was 23.5°.

Fig. 3. Effect of urea concentration on rate of SH appearance. Each cuvette contained 100 μ moles Tris-HCl pH 8.1, 100 μ moles DTNB, urea to give the indicated concentrations, and 100 μ g enzyme in a total volume of 1.0 ml. First-order rate constants were obtained from the slopes of plots similar to those in Fig. 2. Also shown for comparison is the first-order rate constant of the change in absorbancy at 260 $m\mu$ of the enzyme in 7.0 M urea (see Fig. 4 for conditions).

GLL-6411-3286

GLL-6411-3282

Fig. 4. Difference spectrum of enzyme in water and in 7.0 M urea.. Each cuvette contained 100 μ moles Tris-HCl pH 8.1 and 400 μ g enzyme in a total volume of 1.0 ml.

GLL-6411-3285

Fig. 5. Effect of temperature on rate of SH appearance in 4 M urea. The contents of each cuvette are the same as described in Fig. 2 with 100 μg enzyme.

GLL-6411-3284

Fig. 6. Comparison of loss of enzyme activity and appearance of SH. The cuvette contained 100 μ moles Tris-HCl pH 8.1, 100 μ moles DTNB, 200 μ g enzyme in 1.0 ml of 3.8 M urea. The rate of SH appearance was determined in a manner similar to that described in Fig. 2, and aliquots were taken at the indicated times for the determination of enzyme activity (12). Temperature was 26°.

GLL-6411-3283

Fig. 7. Comparison of loss of activity in the presence and in the absence of DTNB. Enzyme (200 μ g) was incubated in 1.0 ml of 4 M urea containing 100 μ moles of Tris-HCl pH 8.1 and either 100 $m\mu$ moles of DTNB or none as indicated. Aliquots were removed for activity measurements (12). Temperature was 26°.

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission " includes any employee or contractor of the commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.