THE IDENTIFICATION OF AN ASYMMETRIC COMPLEX OF NICOTINAMIDE ADENINE DINUCLEOTIDE AND PIG HEART CYTOPLASMIC MALATE DEHYDROGENASE

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SUMMARY: Examination of the two major crystalline forms, type A and type C, of pig heart cytoplasmic malate dehydrogenase (s-MDH) has revealed that type C crystals are composed of enzyme molecules with one mole of nicotinamide adenine denucleotide bound per mole of enzyme. Type A crystals are devoid of bound coenzyme. The NAD^+ can be readily removed by dialysis or gel filtration after type C crystals have been dissolved. The binding of 1 mole of NAD^+ results in the formation of an asymmetric complex in which one of the two identical polypeptide chains composing native s-MDH undergoes a small but definitive conformational change. These data are consistent with observed difference in crystallographic properties of the s-MDH subunits.

Pig heart malate dehydrogenase has been demonstrated to occur in two unique forms (1,2). One form is associated exclusively with the mitochondrion (m-MDH) while the other is found in the cytoplasm (s-MDH). In addition to cellular distribution, these forms may be readily distinguished on the basis of their electrostatic and kinetic properties (3,4). Both enzymes have a dimeric structure composed of apparently identical subunits of about 36,000 molecular weight (5,6). In view of the potential information regarding mechanism of action and control inherent in the study of two enzymes with distinct structures catalyzing the identical reaction, studies to completely define the primary and

three-dimensional structure of both m- and s- MDH have been initiated. In the course of these experiments, a crystal derivative of s-MDH with apparently induced asymmetry has been isolated and characterized. This crystal form contains one mole of NAD^+ per mole enzyme, bound in a non-covalent but stoichiometric manner.

EXPERIMENTAL

Pig heart s-MDH was prepared by a modification of the procedure of Banaszak (7). Type A crystals were prepared from 62% ammonium sulfate solutions buffered in 0.02 M sodium phosphate, pH 6.2 at 4°. Type C crystals were grown from 60-63% saturated ammonium sulfate solutions buffered with 0.05 M sodium acetate, pH 5.1, at 4° (8) containing a 10 fold molar excess of NAD^+ and 0.001 M EDTA. The properties of the crystals and the structure of s-MDH at low resolution, have been described previously (9).

For the determination of NAD^+, solutions of enzyme were prepared by dissolving the appropriate aliquot of washed crystals in 0.02 M phosphate buffer, pH 6.5 containing 0.001 M EDTA and 0.001 M BME and the spectra from 500 to 240 nm recorded on a Cary 15 recording spectrophotometer. The solutions were then brought to 5% in formic acid and incubated overnight at room temperature with pepsin (Worthington Biochemical Corp.) at a weight ratio of 20:1. The NAD^+ content of the peptic hydrolysate was determined by assay with glyceraldehyde-3-phosphate dehydrogenase (Sigma Chemical Co.) as previously described (10). The ratio of NAD^+ to s-MDH was calculated from the known extinction coefficients (11).

RESULTS

The NAD^+ contents of A and C-type crystals as well as the amorphous precipitate of s-MDH, as measured by enzymatic analysis, are summarized in Tables I and II. In Experiment 1 (Table I), C-type crystals were exhaustively washed until the wash solutions were completely free of NAD^+. Determination of the NAD^+ content of this

TABLE I

NICOTINAMIDE ADENINE DINUCLEOTIDE CONTENT OF "C" TYPE CRYSTALLINE PREPARATIONS OF PIG HEART CYTOPLASMIC MALATE DEHYDROGENASE[a]

EXPERIMENT NUMBER	SAMPLE TYPE	TREATMENT	NAD^+/s-MDH[b]
1	"C" Crystals	Washed 4 X with 70% $(NH_4)_2SO_4$, in acetate buffer, pH 5.1	0.94 ± 0.10[c]
2	"C" Crystals	Same as Experiment 1, followed by incubation with 20 mM NAD^+, 20 mM L-Malate, 40 hr. at 4° in same buffer and washed again as in Experiment 1.	1.20 ± 0.12[d]
3	"C" Crystals	Same as Experiment 2 without L-Malate	0.87 ± 0.11[e]
4	"C" Crystals	Same as Experiment 2, followed by dialysis of dissolved crystals against phosphate, pH 6.5	<0.01
5	"C" Crystals	Same as Experiment 2, followed by gel filtration of sample dissolved in:	
		(a) Phosphate, pH 6.5	<0.01
		(b) Phosphate, pH 6.5 and 0.001M NAD^+	<0.01
		(c) Phosphate, pH 6.5 and 0.10M NAD^+	<0.01
6	"C" Crystals	Same as Experiment 1, followed by incubation with NAD^+ solutions, dialysis against 57% $(NH_4)_2SO_4$, in phosphate 6.5, and then precipitation by dialysis against 70% $(NH_4)_2SO_4$, phosphate, 6.5	
		NAD^+ Conc:	
		(a) 10^{-1}M	<0.01
		(b) 10^{-2}M	<0.01
		(c) 10^{-3}M	<0.01
		(d) 10^{-4}M	<0.01
		(e) 10^{-5}M	<0.01
7	"C" Crystals	Same as Experiment 7, except precipitation with saturated $(NH_4)_2SO_4$	
		All NAD^+ Conc:	<0.01

a Measured enzymatically by the method of Ferdinand (10).

b Molecular Weight = 72,000 (7).

d Average of 4 Determinations.

e Average of 2 Determinations.

TABLE II

NICOTINAMIDE ADENINE DINUCLEOTIDE CONTENT OF "A" TYPE CRYSTALLINE AND AMORPHOUS PREPARATIONS OF PIG HEART CYTOPLASMIC MALATE DEHYDROGENASE[a]

EXPERIMENT NUMBER	SAMPLE TYPE	TREATMENT	NAD^+/s-MDH[b]
1	"A" Crystals	Washed 4 X with 70% $(NH_4)_2SO_4$, in phosphate buffer, pH 6.5	0.11 ± 0.01[c]
2	"A" Crystals	Same as Experiment 1, followed by incubation with 20 mM NAD^+ and 20 mM L-Malate, 40 hr, 4° in same buffer and washed again as in Experiment 1 above.	0.82 ± 0.05[c]
3	"A" Crystals	Same as Experiment 2, Table I	0.67 ± 0.04[c]
4	Amorphous Precipitate	Same as Experiment 1	0.05 ± 0.01
5	Amorphous Precipitate	Same as Experiment 2	<0.01

a Measured enzymatically by the method of Ferdinand (10).
b Molecular Weight = 72,000 (7).
c Average of 2 determinations.

sample revealed that it contained 0.94 ± 0.10 moles NAD^+/mole enzyme. Incubation of C-type crystals with large excesses of NAD^+, both in the presence and absence of L-malate (Experiments 2 and 3), did not result in the similar tight binding of a second mole of NAD^+. The slightly higher values observed in Experiment 2 are probably not significant.

In order to ascertain whether this single mole of NAD^+ remained firmly bound to the enzyme after solubilization of the crystals, samples of C crystals were either thoroughly dialyzed (Experiment 4) or passed over a column of Sephadex G-25 (Experiment 5). In Experiment 4, the crystals were dissolved in phosphate buffer, pH 6.5 containing 10^{-3} M EDTA and 10^{-3} M β-mercaptoethanol while in Experiment 5, the crystals were dissolved in solutions of the same buffer and in buffer containing 10^{-1} and 10^{-3} M NAD^+. In each case, no NAD^+ was detectable in the recovered protein.

Experiments to test whether the enzyme could be precipitated by $(NH_4)_2SO_4$ with this mole of NAD^+ bound were also performed. In Experiment 6 (Table I), C-type crystals were dissolved in phosphate buffer pH 6.5 containing five different concentrations of NAD^+. The incubated solutions were then dialyzed against 57% $(NH_4)_2SO_4$ followed by precipitation with 70% $(NH_4)_2SO_4$. In Experiment 8, the precipitation was achieved more rapidly by saturated $(NH_4)_2SO_4$. However, no NAD^+ was detectable in any of these samples.

Somewhat different results were obtained with A-type crystals. As shown in Experiment 1 of Table II, examination of A crystals after exhaustive washing showed only 0.11 moles of NAD^+/mole of enzyme. Thus, in contrast to C-type, this crystalline habit of s-MDH does not contain NAD^+ bound to either subunit of the enzyme molecule. However, as shown in Experiment 2, incubation of this crystal type with larger excesses of NAD^+ at pH 6.5, resulted in the incorporation of 0.82 moles NAD^+/mole of enzyme. No change in the physical

appearance of the crystals was observable under the optical microscope. In contrast, incubation of the A-type crystals at pH 5.1 (as with C-type) with excess coenzyme (Experiment 3) resulted in some incorporation but produced cracking. For the purposes of comparison, amorphous protein does not bind NAD^+, even after incubation with excess NAD^+ (Experiments 4 and 5).

DISCUSSION

The results of these experiments indicate that the two principal crystal habits of pig heart s-MDH differ primarily as a result of their content of bound NAD^+. Clearly, one mole of NAD^+ becomes bound to one of the subunits of the enzyme under conditions leading to crystallization of the C form.

Corroborating evidence for the presence of bound NAD^+ is not obtainable from a low resolution electron density map. However, x-ray studies have been made on the effect of higher concentrations of NAD^+ on s-MDH in the type C lattice (as in Experiment 2, Table I). In this study, type C crystals soaked in high concentrations of NAD^+ were compared to native C crystals using difference Fourier methods (9) One region of electron density, corresponding approximately in shape and size to that expected for a molecule of NAD^+, was visible in a crevice near the surface of one of the two subunits. In view of the experiments described above, this NAD^+ must correspond to a second molecule of bound NAD^+. The results shown in Experiment 2 and 3 (Table I) indicate that this second molecule can be effectively removed by simply washing the crystals. Thus, the affinity of NAD^+ for this second site must be reduced considerably.

These findings are summarized by a schematic representation of the s-MDH molecule shown in Figure 1. The NAD^+ site labelled A corresponds to the binding site identified by x-ray analysis, after soaking C crystals in excess NAD^+ (8). The site labelled B corresponds to the position at which the coenzyme molecule detected

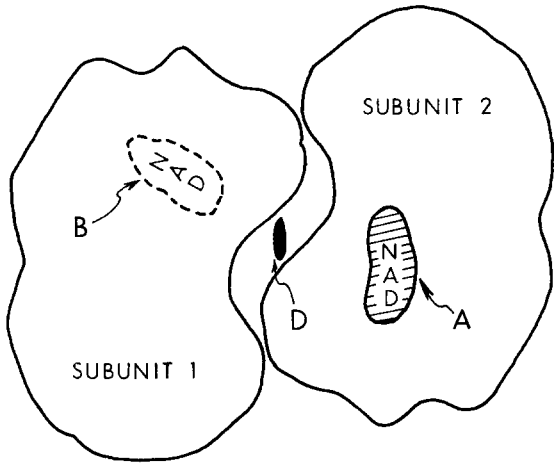

Fig. 1 Schematic representation of the 5.0 Å crystallographic structure of pig heart s-MDH. The NAD^+ site, identified crystallographically, is labelled A. Site B, corresponds the second site, implied by chemical data, but as yet not defined by x-ray results. D represents the approximate two-fold axis of symmetry, relative to the A binding site.

by chemical analysis should be found. A non-crystallographic two-fold symmetry axis relating the two subunits is designated as D. Although the two subunits are apparently sequentially identical (12), the differential affinity of the dimer for NAD^+ suggests that the two-fold rotational symmetry can only be approximate.

Further evidence in support of the conclusion that s-MDH in the C-type crystal habit has conformationally distinguishable subunits has been obtained from the x-ray studied. These observations include the distribution of heavy atom derivatives, particularly p-hydroxy-mercuriphenyl sulfonate, which is located at two sites on one subunit and only one site on the other (9) and the preliminary interpretation of the course of the polypeptide backbone of each subunit in the 3.0 Å electron density map (13).

The relation of the A-type crystal form to that of the C-type is presently unclear. The chemical analysis shows that the A type has a negligible amount of bound NAD^+. Although x-ray diffraction data indicate that the A and C crystals are almost isomorphous, measurable

differences in the intensities of the x-ray data has been observed (9). However, it appears from the results of Experiment 2, Table II, that A-crystals are capable of binding one mole of NAD^+ and that this NAD^+ is not removed by subsequent washing. Whether these results reflect pre-existing conformational differences between subunits or induced differences occurring without destruction of the crystal lattice cannot be distinguished at this time. The possibility that binding occurs to a symmetrical molecule where the second site is blocked by interaction with other molecules of the lattice are thought to be remote (9).

The demonstration of differential binding of s-MDH in C-type crystals clearly indicates the potential of this molecule to function in a non-symmetric manner. However, it is not possible to establish at present, whether this characteristic of s-MDH in the crystalline state reflects a similar property of the enzyme in solution. Experiments to ascertain whether this property of s-MDH represents a regulatory or mechanistic feature of s-MDH catalysis, such has been observed with other enzymes (14,15), is currently under investigation.

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REFERENCES

1. R. G. Wolfe and J. B. Nielands, J. Biol. Chem., 221, 61 (1956).
2. C. J. R. Thorne and P. M. Cooper, Biochim. Biophys. Acta, 81, 397 (1963).
3. C. J. R. Thorne and N. O. Kaplan, J. Biol. Chem., 238, 1861 (1963).
4. C. J. R. Thorne, L. I. Grossman and N. O. Kaplan, Biochim. Biophys. Acta, 73, 193 (1963).
5. T. Devenyi, S. J. Rogers and R. G. Wolfe, Nature, 210, 489 (1966).
6. R. K. Gerding and R. G. Wolfe, J. Biol. Chem., 244, 1164 (1969).
7. L. J. Banaszak, J. Mol. Biol., 22, 389 (1966).
8. L. J. Banaszak, D. Tsernoglou and M. J. Wade, "Probes of Structure and Function of Macromolecules and Membranes", Vol. II, Academic Press, Inc., New York (1971) p. 71.

9. D. Tsernoglou, E. Hill and L. J. Banaszak, Cold Spring Harbor Symp. Quant. Biol., XXXVI (1971) in press.
10. W. Ferdinand, Biochem. J., 92, 578 (1964).
11. M. J. Wade, Ph.D. Thesis, Washington University (1971).
12. R. A. Bradshaw, B. E. Glatthaar and M. J. Wade, unpublished experiments.
13. D. Tsernoglou, L. Webb, E. Hill and L. J. Banaszak, unpublished experiments.
14. M. Lazdunski, C. Petitclerc, D. Chappelet, and C. Lazdunski, Eur. J. Biochem, 20, 124 (1971).
15. A. Levitzki, W. B. Stallcup, and D. E. Koshland, Jr., Biochemistry, 10, 3371 (1971).