Crystalline Symmetry and Coenzyme Binding Properties of D-Glyceraldehyde-3-phosphate Dehydrogenase from the Tail Muscle of Palinurus vulgaris*

Rodolfo Berni, Andrea Mozzarelli, and Gian Luigi Rossi†

From the Institute of Molecular Biology, University of Parma, Cornocchio, 43100 Parma, Italy

Martino Bolognesi and Roberta Oberti

From the Institute of Crystallography, University of Pavia, Via Bassi 4, 27100 Pavia, Italy

Crystals of apo- and holo-D-glyceraldehyde-3-phosphate dehydrogenase from the tail muscle of the Mediterranean lobster Palinurus vulgaris, previously found to be suitable for single crystal microspectrophotometric studies of catalytic activity in the crystalline state, have been examined by x-ray crystallography. The two forms are isomorphous, space group C 2 with cell dimensions a = 128.4 Å, b = 99.9 Å, c = 80.3 Å, β = 113.4°. These data are consistent with a molecular weight of 73,000 in the crystallographic asymmetric unit, indicating that the tetrameric molecule possesses an exact 2-fold axis both in the presence and in the absence of NAD+. Analysis of the intensity distribution of conventional x-ray precession photographs shows that two further noncrystallographic diads are present and that the molecule has the 2 pseudo 22 symmetry found in other D-glyceraldehyde-3-phosphate dehydrogenases. Binding of NAD+ to apoenzyme in solution, at 25°C, is anticohesive and can be satisfactorily described by assuming two classes of coenzyme binding sites.

Glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12), an enzyme composed of four chemically identical subunits that catalyzes the NAD+-dependent oxidative phosphorylation of D-glyceraldehyde-3-phosphate to 1,3-diphosphoglyceric acid, has been isolated and crystallized from various sources (1) and high resolution structures have been reported for holoenzyme from the tail muscle of the Atlantic lobster Homarus americanus (2, 3) and from Bacillus stearothermophilus (4).

In solution, both enzymes exhibit negative cooperativity in coenzyme binding (5, 6), a phenomenon described on the basis of two parameter models: ligand-induced sequential changes in a "a priori" symmetric molecule (7-9) and pre-existing sites heterogeneity (10-13). The molecular symmetry of apo- or holoenzyme from either source is not directly expressed in the crystal; however, the lobster holoenzyme shows possible pairwise asymmetry in the 2.0 Å resolution electron density map (14), whereas the R. stearothermophilus holoenzyme is reported to possess accurate 222 symmetry in the 2.7 Å resolution electron density map (4). Molecular asymmetry in the case of the lobster enzyme was further confirmed by the crystallographic study of an abortive ternary complex (15).

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† To whom all correspondence should be addressed.

In this communication, we report crystallographic data for holo- and apoglyceraldehyde 3-phosphate dehydrogenase from the tail muscle of the Mediterranean lobster Palinurus vulgaris, an enzyme previously shown to have similar function-related properties in the crystal and in solution (16, 17). We also report data for the binding of NAD+ to apoenzyme in solution.

Enzyme from the tail muscle of P. vulgaris was purified and crystallized as previously described; its specific activity was usually 300 ± 10 units/mg (16).

Holoenzyme crystals of suitable size were examined by means of common precession photographs (Fig. 1). Very strong quasiorthorhombic symmetry could be observed, but accurate analysis of symmetry-related reflections showed a breakdown of mm symmetry. The lattice was indexed on the basis of monoclinic cell with β = 113.4°; the systematic absences showed the space group to be C 2 with cell dimensions a = 128.4 Å, b = 99.9 Å, c = 80.3 Å. The value of the Vm parameter corresponding to this molecular packing is 3.26 Å3/dalton and 60% of the crystal volume is occupied by the solvent (18). Assuming a molecular weight of 146,000 for the tetrameric molecule, the asymmetric unit can accommodate only a mass of 73,000 daltons, i.e., the molecule possesses an exact 2-fold axis coincident with the crystallographic b axis. Inspection of upper levels, in particular (h01) and (n,k,l) with n odd, showed, on occasions, doubling of c*. As in the case of human holo-glyceraldehyde-3-phosphate dehydrogenase, this could be explained by a twinning axis perpendicular to the ab plane (19). The analogy with the human enzyme and, furthermore, with the crayfish enzyme (20), is enhanced by the strong similarity of the intensity distribution of the corresponding precession photographs (compare Fig. 1a with Fig. 3c of Ref. 19 and Fig. 3b of Ref. 20). The mm extra symmetry observed in the (h01) layer of human, crayfish, and P. vulgaris holo-glyceraldehyde-3-phosphate dehydrogenase crystals indicates that in all cases the enzyme possesses two additional local 2-fold axes, lying at right angles to the exact molecular diad; in the human enzyme, the latter has been shown to be the Q 2-fold axis (2, 19). On the basis of the above observations (i.e., space group identity, comparable unit cells, very similar diffraction patterns with strong mm symmetry), it is conceivable that also in P. vulgaris holo-glyceraldehyde-3-phosphate dehydrogenase, the Q molecular diad corresponds to the crystallographic axis b, while the local and inexact 2-fold axes found in the (h01) layer represent the P and R molecular pseudo-diads observed in the H. americanus holoenzyme (14).

Asymmetry between the R axis-related subunits, whose active sites are spatially interacting (2), has been considered a possible structural basis for the enzyme cooperativity (2, 14) and
A Triosephosphate Dehydrogenase with Exact 2-fold Symmetry

**Fig. 1. Precession photographs.**

a. Precession photograph of holo-β-glyceraldehyde-3-phosphate dehydrogenase from *Palinurus vulgaris*. Precession angle, 12.5°; c* is vertical; crystal to film distance, 60 mm. b. Precession photograph of apo-β-glyceraldehyde-3-phosphate dehydrogenase from *Palinurus vulgaris*. Holo zone, precession angle 7°; crystal to film distance, 60 mm.

It has been shown that the ternary complex glyceraldehyde-3-phosphate dehydrogenase-trifluoroacetone-nicotinamide adenine dinucleotide (15).

Since coenzyme binding is expected to cause molecular changes, it is of interest to compare crystals of apo- and holo-forms of the enzyme. No adequate structural data have as yet been presented to decide whether 2 pseudo 22 molecular symmetry might exist even in apoglyceraldehyde-3-phosphate dehydrogenase.

*P. vulgaris* apoenzyme was obtained by removing NAD⁺ from holoenzyme microcrystals that were repeatedly washed with acidic ammonium sulfate solutions (pH 3.6) and finally dissolved. Apreparation of crystals was carried out at 4°C, in small glass test tubes, where an enzyme solution (12 mg/ml of protein, 30% saturated ammonium sulfate, 1 mM EDTA) was layered on top of an equal volume of solution containing 80% saturated ammonium sulfate, 1 mM EDTA, 0.75 mM dithiothreitol, pH 6.2. The NAD⁺ remaining bound to the crystalline enzyme was estimated after dissolving the crystals, either on the basis of the A₆₀₀/A₅₀₀ ratio or on the basis of the fluorescence of reduced coenzyme, produced in the presence of excess β-glyceraldehyde-3-phosphate and arsenate (12), and was found to be lower than 0.1 mol/mol of tetramer.

When apoenzyme crystals were soaked in a 70% saturated ammonium sulfate solution containing 1 mM EDTA and NAD⁺ in the concentration range between 10⁻⁸ M and 10⁻⁴ M, coenzyme binding occurred, as indicated by the appearance of the Racker band, although at a much higher coenzyme concentration than expected from solution studies (see below). However, numerous fine cracks could be noticed. This observation suggests that definite molecular changes must accompany NAD⁺ binding.

Comparison of precession photographs of holo- and apoenzyme crystals showed that they are isomorphous and that local asymmetry may be present in the apoenzyme molecule. Some differences in the intensity distribution of apo- and holoenzyme precession photographs could be detected.

The observed 2-fold crystallographic symmetry requires that no more than two classes of NAD⁺ binding sites pre-exist within the apoenzyme tetramer and, likewise, that the coenzyme-occupied sites within the holoenzyme be identical at least in pairs.

In order to relate this structural feature with functional properties of the enzyme, we investigated NAD⁺ binding of *P. vulgaris* glyceraldehyde-3-phosphate dehydrogenase in solution, both in ethylenediamine buffer (10 mM ethylenediamine, 1 mM EDTA, 100 mM KCl, pH 7) and in a high salt medium, similar to the crystals mother liquor, containing 50% saturated ammonium sulfate, 1 mM EDTA, pH 7. The isotherms of NAD⁺ binding to apoenzyme were determined fluorimetrically, as previously described (12, 21, 22), at 25°C.

The concave Scatchard plot of the data (Fig. 2) and the Hill coefficient, n = 0.77, indicate that NAD⁺ binding to the apoenzyme in ethylenediamine buffer is an anticooperative process (23). As previously found for glyceraldehyde-3-phosphate dehydrogenase isolated from sturgeon muscle (12), *B. stearothermophilus* (6) and rabbit muscle, at least according to a recently reported procedure (24), the data can be satisfactorily fitted to a general two-sites Adair equation. The phenomenological dissociation constants are: K₁ = 5.2 × 10⁻⁸ M and K₂ = 9.9 × 10⁻¹⁰ M, respectively, for the two tight and the two loose sites. In the presence of 50% saturated ammonium sulfate, NAD⁺ binding is apparently tighter than in ethylenediamine buffer. However, even in this medium, NAD⁺ binding can be described on the basis of two classes of sites with dissociation constants K₁ = 3.3 × 10⁻⁸ M and K₂ = 3.3 × 10⁻¹⁰ M. It is therefore conceivable that the negative cooperativity in coenzyme binding be associated with pre-existing 2 pseudo 22 molecular symmetry, as previously discussed (12, 13).

We also tried to estimate dissociation parameters for NAD⁺ bound to the crystalline enzyme. Defined amounts of holoenzyme crystals were transferred into variable volumes of a solution containing 70% saturated ammonium sulfate and 1 mM EDTA, pH 7. This dilution-titration procedure allowed us to determine the fractional saturation of crystalline glyceraldehyde-3-phosphate dehydrogenase (at equilibrium after the progressive release of NAD⁺) on the basis of the A₆₀₀/A₅₀₀ ratio values of the collected and dissolved crystals. Only 1 NAD⁺ molecule dissociates from the crystalline enzyme with a measurable dissociation constant (K = 10⁻¹⁰ M). Similarly, in the case of *B. stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase, crystallographic observation allowed others to establish that NAD⁺ was preferentially released by one site within the tetramer, probably due to the molecular

![Fig. 2. NAD⁺ binding isotherms for *P. vulgaris* β-glyceraldehyde-3-phosphate dehydrogenase.](image-url)

**Fig. 2. NAD⁺ binding isotherms for *P. vulgaris* β-glyceraldehyde-3-phosphate dehydrogenase.** Scatchard plot of the enzyme fluorescence quenching data. Y is the saturation function. Free NAD⁺ concentrations are calculated on the basis of the stoichiometry of 4 molecules of NAD⁺ bound/enzyme molecule and of an equal contribution of each site to the observed fluorescence quenching. The solid lines are calculated from a general two sites Adair equation using the dissociation constants indicated in the text. Conditions: A, 0.028 μM enzyme, ethylenediamine buffer (10 mM ethylenediamine, 1 mM EDTA, 100 mM KCl), pH 7, 25°C; B, 0.028 μM enzyme, 50% saturated ammonium sulfate, 1 mM EDTA, pH 7, 25°C.
packing within the crystal (25). These findings suggest that molecular interactions within the holoenzyme crystal interfere with the structural transitions normally associated with coenzyme release and prevent the dimer of dimers behavior exhibited by the enzyme in solution.

In contrast, we reported that lattice forces do not affect the “half-of-the-sites-reactivity” of holoenzyme in the reaction with the substrate-analog, β-(2-furyl)acryloyl phosphate, nor the NAD⁺-activated arsenolysis of the acyl-enzyme; furthermore, NAD⁺ could be completely removed from the diacylated enzyme without causing crystal shattering (16, 17). From present and previous data, we therefore conclude that *P. vulgaris* glyceraldehyde-3-phosphate dehydrogenase functions as a dimer of dimers both in solution and in the crystalline state and that it contains two classes of NAD⁺ binding sites. Although, in the crystalline state, lattice forces do not allow the functional expression of this property. As previously noted (26), no glyceraldehyde-3-phosphate dehydrogenase has yet been found to possess maximal crystallographic symmetry, while all the data, with one exception (4, 25), are consistent with the “exact dimer of inexact dimers” hypothesis (27).

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REFERENCES