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Histochemical method for characterization of enzyme crystals: application to crystals of *Torpedo californica* acetylcholinesterase

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Histochemical methods are employed to detect and localize a wide range of enzymes. Even though protein crystallographers do not commonly use this technique, the extensively used colorimetric reaction of Karnovsky was successfully adapted for easy and quick identification of acetylcholinesterase crystals. The method relies on the reduction of ferricyanide to ferrocyanide by thiocholine, released from acetylthiocholine by enzymatic hydrolysis, followed by formation of a cupric ferrocyanide precipitate, and allows rapid differentiation between salt and enzyme crystals and between native and inhibited crystals of the enzyme.

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1. Introduction

Protein crystallographers often need to identify the content of a crystal. At the early stage of searching for crystallization conditions, it is necessary to know whether a crystal is made of protein or of salt. At the later stage of understanding structure–function relationships, the most common procedure is to obtain a crystal of an enzyme–inhibitor complex, either by soaking the native crystal in a solution containing the inhibitor or by cocrystallizing the inhibitor with the native enzyme. Thus, the question asked is: ‘does the crystal contain the inhibited form of the enzyme?’ In both cases, X-ray diffraction data can provide the answer. Initially, to tell if a crystal is salt or protein, it is often possible to distinguish a salt crystal from that of a protein by just touching one such crystal in question. If it is a protein it is usually soft and if it is a salt it is very hard. However, for very small crystals, *e.g.* <0.1 mm, this ‘touching’ method is often just not feasible and/or conclusive. Therefore, a single X-ray diffraction frame is needed. However, to establish that a crystal indeed contains the inhibited form of the enzyme, rather than the native enzyme, analysis of the initial Fourier difference map from a full data set is necessary.

The structures of native *T. californica* acetylcholinesterase (*TcAChE*; Sussman *et al.*, 1991) and of various complexes and conjugates with, respectively, reversible and covalent inhibitors (Greenblatt *et al.*, 1999; Harel *et al.*, 1993; Kryger *et al.*, 1999; Millard *et al.*, 1999; Raves *et al.*, 1997) have been solved by X-ray crystallography. Even though many insights into key structural elements of the catalytic and anionic binding sites were obtained, the localization of the binding sites for divalent

ions such as magnesium, calcium and manganese would help to elucidate their putative structural and functional roles. Such divalent cations have been shown to affect the kinetic, ligand-binding and physicochemical characteristics of AChE (see, for example, Changeux, 1966; Foidart & Gridelet, 1974; Pattison & Bernhard, 1978; Wins *et al.*, 1970).

Initial attempts to crystallize native *TcAChE* in the presence of manganese ions produced tiny crystals which were too small to be characterized even at a powerful synchrotron source. It was decided, therefore, to employ a commonly used histochemical method, the Karnovsky procedure (Karnovsky & Roots, 1964), to determine if these crystals were protein or salt.

2. Materials and methods

2.1. Purification and crystallization of *TcAChE*

TcAChE was purified as described by Futerman *et al.* (1985). The enzyme was concentrated to 10–13 mg ml⁻¹ in 0.3 M MES, 100 mM NaCl, 0.02% NaN₃ pH 5.8.

Crystallization of *TcAChE* in the presence of manganese ions was performed at 293 K. Hanging drops were obtained by mixing 2 µl of enzyme with an equal volume of crystallization solution containing 10–15% PEG 5000, 0.2 M manganese (II) acetate, 0.1 M MES pH 6.3. Tiny crystals appeared within 5 d and did not grow further.

For studies with ENA-713, which inhibits the enzyme irreversibly by carbamylation of its active-site serine (Bar-On *et al.*, 1998, 2000), trigonal crystals of native *TcAChE* were obtained after two weeks at 277 K with hanging drops consisting of 3 µl of *TcAChE*

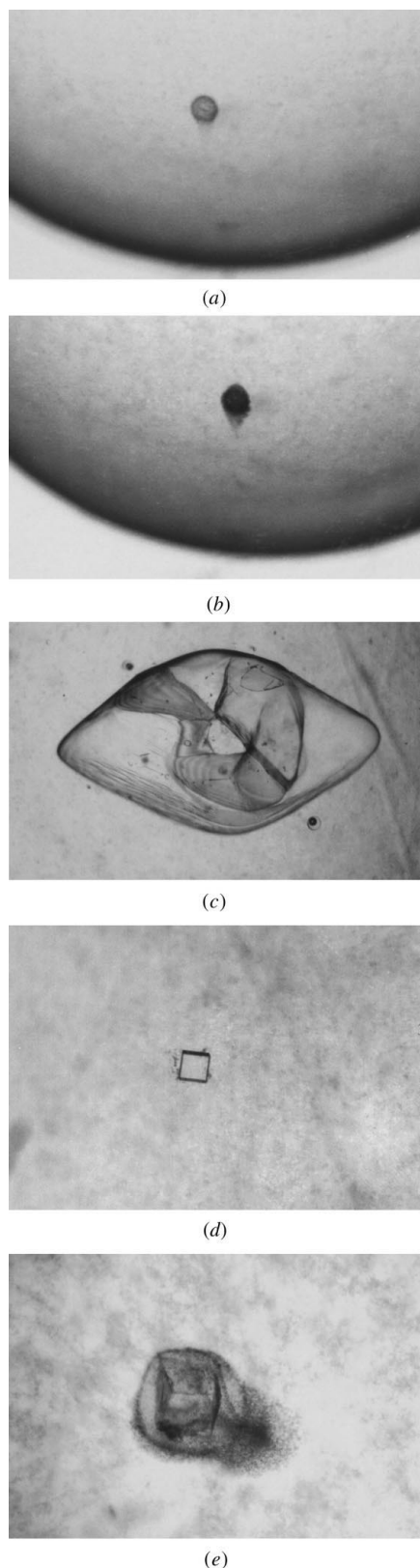


Figure 1

Crystals in the Karnovsky solution containing acetylthiocholine: tiny crystal of *TcAChE* grown in the presence of manganese (II) acetate after (a) 5 min and (b) 15 min; (c) manganese (II) acetate crystal; (d) trigonal crystals of the complex *TcAChE*-ENA and (e) of native *TcAChE*.

and 3 μ l of 33% PEG 200, 0.1 M MES pH 5.7, equilibrated with a well containing 40% PEG 200. Such crystals were soaked in 3 mM ENA-713, 33% PEG 200, 0.1 M MES pH 5.7 for 13 d.

2.2. Histochemical staining

With a nylon cryoloop (Hampton Research), the crystal was fished from its harvesting drop and first transferred into 5 μ l of mother liquor in order to remove any traces of soluble enzyme. It was then transferred to 3 μ l of mother liquor containing 3 mM CuSO_4 and 0.5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ (both purchased from Merck). After incorporation into the drop of 2 μ l of 50 mM acetylthiocholine iodide (Sigma), a black precipitate appeared around the crystal within 5 min.

3. Results

3.1. Differentiation between salt and enzyme crystals

When a tiny crystal of *TcAChE* grown in the presence of manganese ions was washed and transferred to the Karnovsky solution, after 5 min a black precipitate could be observed around the crystal (Figs. 1a and 1b).

As a control, a manganese (II) acetate crystal was transferred to crystallization mother liquor containing the Karnovsky reagents and acetylthiocholine. No black precipitate could be detected (Fig. 1c).

3.2. Differentiation between catalytically inactive and active *TcAChE* crystals

A crystalline conjugate of ENA-713 with *TcAChE* was obtained by soaking trigonal crystals of native *TcAChE* in 3 mM ENA-713. Such a crystal was washed and transferred into the Karnovsky solution. No black precipitate could be observed even after 30 min and after several additions of 2 μ l of acetylthiocholine to the drop (Fig. 1d).

In contrast, a crystal of native *TcAChE* grown under the same conditions (Sussman *et al.*, 1988) and soaked in the Karnovsky solution produced a black precipitate within 2 min of addition of 2 μ l of acetylthiocholine (Fig. 1e).

4. Discussion and conclusions

The data presented above show that a suitable histochemical method can provide a simple and rapid procedure both for identifying an enzyme crystal and for assessing its

catalytic status. Such an approach will be of general applicability to a broad spectrum of enzymes for which histochemical procedures have been described (see Burstone, 1962; Pearse, 1980; Thompson, 1966). Previously described methods using protein dyes both lack specificity and do not produce significant coloration of the tiny crystals which can now be utilized at third-generation synchrotrons. In contrast, the signal in the histochemical procedure is both specific and amplified by the catalytic reaction.

Thus, the Karnovsky procedure was able to readily distinguish between a crystal of *TcAChE* and a crystal of an inorganic salt which, since it was included in the mother liquor, could well have precipitated in the hanging drop.

Clear-cut evidence that, in soaking experiments, a given inhibitor has penetrated into an enzyme crystal and either bound to or reacted with the active site usually requires collection of a complete medium- to high-resolution data set and its subsequent refinement. In the case which we chose to study, the anti-Alzheimer's drug, ENA-713, which exerts its action by irreversibly carbamylating the active-site serine Ser200 of *TcAChE* (Bar-On *et al.*, 1998, 2000), was soaked into a native trigonal crystal (Sussman *et al.*, 1988) of the enzyme. Examination of the refined structure revealed high occupancy of the carbamyl moiety at the active site, within covalent bonding distance of Ser200 O γ . Since carbamylation is essentially irreversible, it is reasonable to expect that the prolonged soaking period (13 d) would result in complete inhibition, as is borne out by the fact that the Karnovsky procedure did not produce detectable precipitate. It should, however, also be possible to qualitatively detect even partial inhibition. In order to do so, however, it would be necessary to compare the formation of the histochemical precipitate for native and inhibited crystals of similar dimensions.

It is also implicit that a histochemical procedure can be used to check whether a given crystal form of an enzyme retains its catalytic activity.

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