HALOTHANE BINDS IN THE ADENINE-SPECIFIC NICHE OF CRYSTALLINE ADENYLATE KINASE

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1. Introduction

In the past, the action of anaesthetics was generally interpreted in terms of rather unspecific hydrophobic interactions between drugs and biological structures (for a review see ref. [1]). Recent results, however, suggest the existence of specific binding sites for compounds like xenon [2,3] and halothane [4,5]. The nature of halothane receptors might be elucidated by investigating the molecular basis of malignant hyperthermia [6]. This fatal syndrome which some individuals develop in response to halothane anaesthesia is characterized, among other findings, by muscle rigidity and a continuous rise of the body temperature. In several cases halothane-induced malignant hyperthermia was found to be associated with adenylate kinase (EC 2.7.4.3) deficiency [7]. Furthermore, a direct effect of the anaesthetic on the structure and function of this enzyme, which catalyzes the reaction ATP + AMP $\rightleftharpoons$ 2ADP, was demonstrated by various methods [8–10].

These observations prompted us to investigate the binding of halothane to crystals of adenylate kinase by X-ray diffraction analysis. It was found that halothane does not bind to all accessible hydrophobic regions of the protein, but only to the niche which has been identified as the binding site of the adenine moiety of AMP [11].

2. Materials and methods

The substrates and the coupling enzymes used in the assay for adenylate kinase were obtained from Boehringer, Mannheim, halothane from Hoechst, Frankfurt and all other reagents from Merck, Darmstadt.

For experimental reasons adenylate kinase from pig muscle was chosen which is almost identical with the human enzyme [12]. Moreover, malignant hyperthermia is common in pigs [13]. Crystals of adenylate kinase [14] were prepared as previously described [14,15]. In order to bind halothane the enzyme crystals were soaked at 4°C for 1–2 days, at pH 6.1, in 0.1 M Tris–maleate/3 M $(NH_4)_2SO_4$ saturated with halothane.

X-Ray diffraction data of the crystals were collected to a resolution of 0.6 nm using a method described by Schulz et al. [15]. Because of crystal instability higher resolution could not be obtained. The location of halothane on the molecule was derived reproducibly from a difference-Fourier map [16] on the basis of the known protein structure [14]. Part of this map is shown in fig.1.

In kinetic experiments the activity of adenylate kinase was measured at 25°C in a coupled enzyme assay determining the production of ADP [17,18]. A 3 ml assay mixture, of pH 6.5, contained 0.1 M Tris–maleate, 3 mM $MgCl_2$, 350 μM phosphoenolpyruvate, 180 μM NADH, 10 units pyruvate kinase, 10 units lactate dehydrogenase and rate limiting amounts of human or porcine adenylate kinase. In all experiments in which the concentration of the substrate AMP was higher than 500 μM, 2.5 mM halothane had no effect on the enzyme activity; this was true for high (1 mM) and for low (20 μM) concentrations of ATP. However, in the presence of 100 μM AMP (and 1 mM ATP) 2.5 mM halothane

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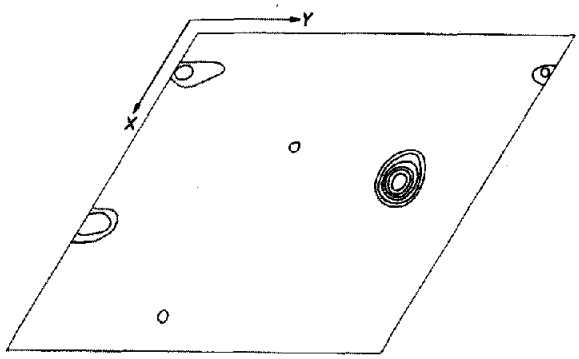

Fig.1. Difference-Fourier map of adenylate kinase soaked with halothane minus adenylate kinase. The enzyme is in the B-conformation [14], space group $P3_121$, crystal, axes $a = b = 4.85$ nm, $c = 13.9$ nm. The map section is $z = 8/60$. Contours are drawn at 40%, 50%,... 90% of maximum density in the map. Only the main peak is significantly above the noise level. Taking into account the density of the underlying protein map, the atomic number difference between halothane and protein and the intrinsic reduction factor 0.5 of a difference-Fourier [16], the width and height of the peak correspond to more than 50% occupancy by halothane.

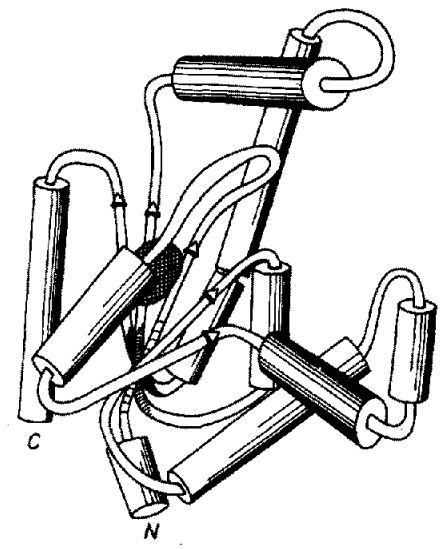

Fig.2. Sketch of adenylate kinase with a bound halothane molecule (hatched ellipsoid). The atomic structure of the enzyme is known [24,25]. Strands of the central β-pleated sheet are represented by arrows and α-helices by cylinders. Halothane binds in a hydrophobic pocket which has been identified as the binding-site of the adenine moiety of the substrate AMP [11].

caused 50% inhibition of adenylate kinase; this inhibitory effect could be reversed by evaporating the anaesthetic.

3. Results and discussion

The difference-Fourier map (adenylate kinase soaked with halothane minus adenylate kinase) contains one significant peak (fig.1). All other peaks of the map exceed the general noise level only slightly. The peak size is about 0.8 X 0.7 X 0.5 nm which corresponds roughly to the dimensions of a halothane molecule ($BrClCH{-}CF_3$) at 0.6 nm resolution. From the width and the height of the peak it was estimated that at least every second crystalline enzyme molecule contains a specifically-bound halothane molecule.

The halothane binding site is a hydrophobic pocket deep in the protein molecule. The location of this site with respect to the general chain fold is illustrated in fig.2. In earlier experiments [11], this pocket was identified as the binding of the adenine moiety of AMP. At the obtainable resolution no details of the interaction between halothane and protein side chains are perceptible; it is, however, known that the pocket is lined by the side chains of Cys-25 as well as Ile-11, Val-13, Ser-19, Gly-20, Gln-24, Ile-28, Leu-91, Leu-116, Leu-118 and Val-186.

As halothane occupies the position of the substrate AMP, the crystallographic results suggest that the anaesthetic affects the enzyme activity. This is in agreement with the findings of Schmidt and his collaborators [9,10] as well as with the kinetic experiments described above. Only when AMP was the variable substrate a reversible inhibitory effect of halothane on human and procine adenylate kinase was observed.

The conditions used in vitro (2.5 mM halothane, 100 μM AMP, pH 6.5 etc) can be assumed to be in the same range as the corresponding conditions in the muscle cells of an anaesthesized person [19]. Consequently our results support Schmidt's hypothesis on the aetiology of halothane-induced malignant hyperthermia [9,10]. According to Schmidt malignant hyperthermia ensues when inherited adenylate kinase deficiency is aggravated by the inhibitory effect of

halothane during anaesthesia. In the absence of sufficient adenylate kinase activity, the ratio of [ATP] : [ADP] : [AMP] in the cell cannot be regulated any longer [17] which is assumed to precipitate the observed sequence of metabolic derangements in malignant hyperthermia. It should be stressed that this fatal syndrome might not be associated with adenylate kinase deficiency in all cases; other aetiologies have been proposed as well [21–23].

4. Conclusion

Adenylate kinase may serve as a model for biological structures in which a purine binding site is affected by halothane (fig.1). It is indeed remarkable that all proteins for which specific interactions with halothane have been demonstrated [19] process functionally important binding sites for purine nucleotides; examples are microtubules [20], glutamate dehydrogenase [4], calcium-transport ATPases [5,21,22] and phosphoryl transferases [10,23]. This aspect could facilitate the search for the principal halothane receptors in normal anaesthesia.

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