European Urology

Eur Urol 2000;37(suppl 3):24–33

Keyhole Limpet Hemocyanin: Molecular Structure of a Potent Marine Immunoactivator

A Review

J. Robin Harris Jürgen Markl

Institute of Zoology, University of Mainz, Germany

Key Words

Keyhole limpet hemocyanin · Structure · Biochemistry · Electron microscopy

Abstract

Objectives: In this short review we present a survey of the available biochemical and electron microscopic data on keyhole limpet hemocyanin (KLH). ***Results:*** The biosynthesis of KLH and its biological role are discussed and the purification of the two isoforms of KLH (KLH1 and KLH2) presented in some detail. The determination of the molecular mass of KLH, its functional unit structure, carbohydrate content, immunological analysis and aspects of the molecular biology of KLH are all dealt with. Transmission electron microscopy (TEM) and crossed immunoelectrophoresis have played a significant part in the understanding of KLH structure. We present a summary of TEM studies on the native oligomers of KLH, the experimental manipulation of the different oligomeric states, immunological analysis and subunit reassociation. ***Conclusion:*** This fundamental structural information provides the scientific background upon which the understanding of the in vivo immunostimulatory function of KLH can be based.

Introduction

Biomedical interest in the high molecular mass hemocyanin of the marine mollusc, the giant keyhole limpet *Megathura crenulata* (commonly abbreviated as KLH), goes back more than 30 years, to the time when this copper-containing extracellular respiratory protein was first found to possess remarkable immunostimulatory properties in experimental animals and man [1–4].

The ready availability of *M. crenulata*, native to the Pacific coast of California and Mexico, the relatively large volume of hemocyanin-rich hemolymph that can be obtained from individual animals (compared to many other small marine and terrestrial molluscs) and the simple fact that in earlier studies this rather than any other hemocyanin was used, rapidly led to the inclusion of a research grade KLH product within the lists of a number of chemical suppliers. Subsequently, there followed the establishment of large-scale processing facilities for the production of a safe KLH subunit clinical product (Biosyn Arzneimittel GmbH, Fellbach, Germany; Immucothel®/Immunocyanin). In addition, the Biosyn Company market the KLH subunit pharmaceutical cGMP grade product Vacmun®, specifically as a carrier for vaccines and antigens.

Despite these advances, fundamental knowledge of the protein structure of KLH has been rather slow. Senozan et

KARGER

Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0302–2838/00/0379–0024$17.50/0

Accessible online at:
www.karger.com/journals/eur

Dr. J. Robin Harris
Institute of Zoology, University of Mainz
D–55099 Mainz (Germany)
Tel. +49 6131 393158/392314, Fax +49 6131 394652
E-Mail rharris@uzomai.biologie.uni-mainz.de

al. [5] observed two different subunit types, but the fundamental fact that there are indeed two independent oligomeric isoforms of KLH emerged much later [6–8]; the currently used terminology 'KLH1 and KLH2' was introduced by Gebauer et al. in 1994 [8], although the isoform terminology KLH-A/KLH-B has also been proposed [9]. Progress through the 1980s and early 1990s has been steady, if slow, with a marked acceleration in the quantity of both fundamental and clinically applied data published throughout the past 5 years. It is the intention of this short review to expand upon these topics from a biochemical and electron-microscopic point of view. Throughout this review it will be seen that considerable interplay between these techniques is emphasized; indeed, the two approaches are mutually supportive.

Biosynthesis of KLH

In gastropods hemocyanin biosynthesis occurs in the 'pore cells', characteristically large cells with much endoplasmic reticulum and a distinctive fenestrated/pore surface, found widely dispersed throughout the connective tissues. Within these cells hemocyanin has been found to accumulate within the endoplasmic reticulum cisternae as intact cylindrical macromolecules and even to form intracellular crystalline arrays [10, 11]. In tissues taken from the keyhole limpet, pore cells have also been detected; however, it has not yet been possible to define KLH molecules within these cells. The difficulty encountered here, with respect to the in situ visualization of KLH, appears to be in accordance with the difficulties faced during attempts to isolate hemocyanin-specific messenger RNA from the tissues of animals taken freshly from the Pacific ocean and from captive animals held for a period of weeks in a salt water aquarium (see below). Initially it appeared that hemocyanin biosynthesis in the keyhole limpet may be rapidly halted by subjecting the animals to the stress of removal from their natural environment. However, we now have reasons to believe that in this animal hemocyanin may be actively synthesized for very short periods, with long resting periods in between.

Biological Role and the Copper-Containing Functional Units of KLH

Hemolymph oxygen uptake, transport and release is the function of the extracellular hemocyanins, all of which are known to be oligomeric assemblies of one or more subunit types [12]. For the molluscan hemocyanins, each functional unit (FU) within the protein subunit contains a binuclear copper binding site that can bind molecular oxygen (O_2). The copper is in a cuprous state and oxygen binding generates light absorbance in the near ultraviolet around 340 nm and with a broad peak around 570 nm, responsible for the characteristic blue color of oxygenated hemocyanins. The peptide sequence surrounding the 2 copper-binding sites (termed A and B) is highly conserved, with 3 copper-liganding histidines in both cases. For *Octopus* hemocyanin FU *g*, which is structurally similar to other molluscan hemocyanin FUs, the X-ray structure has been obtained [13]; it is shown in figure 1.

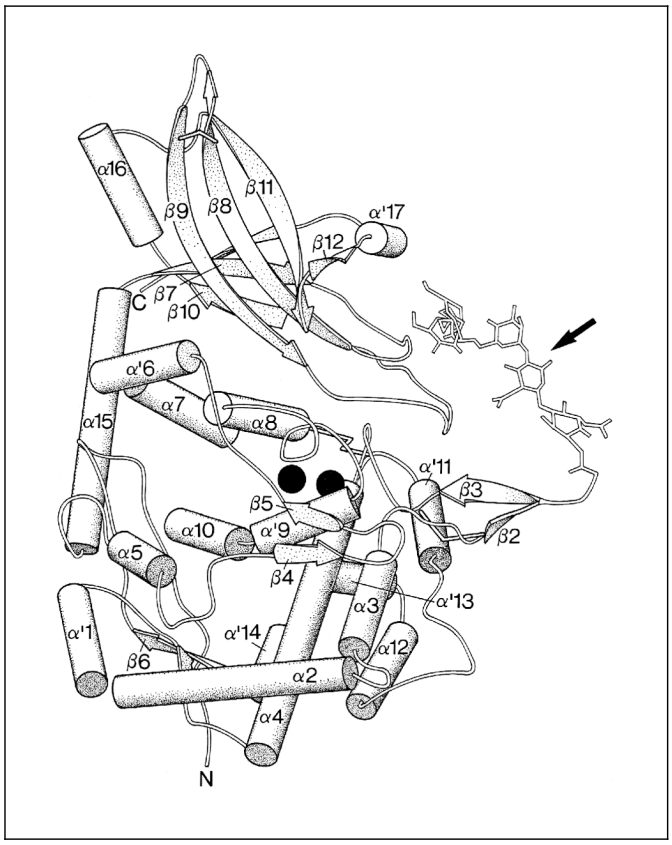


Fig. 1. The X-ray structure of *Octopus dofleini* hemocyanin unit FU *g*, the only currently available high-resolution three-dimensional data from a molluscan hemocyanin. The location of the two copper atoms is indicated by the dark spheres. The carbohydrate side chain is indicated by the arrow. Redrawn from Cuff et al. [13].

Progress on the relationship between hemocyanin oligomer formation and reversible oxygen-binding behavior of KLH is still at an early stage [9]. In view of the recent availability of the two isoforms in highly purified and

ultrastructurally defined oligomerization states, it can be anticipated that an increasing number of physiological and biophysical studies using KLH will be undertaken in the near future.

Purification and Oligomeric States of KLH1 and KLH2

Purification of KLH is routinely monitored by analytical nondenaturing native gel electrophoresis of the dissociated subunits of KLH1 and KLH2 (SDS-PAGE is not useful here, because the molecular mass of the two subunits is very close: approx. 390 kD for KLH1 vs. approx. 360 kD for KLH2 [14]). Preparative native electrophoresis has been used to purify the two subunits [6, 15], but most efforts have been directed towards the purification of the intact molecules. The ability to assess the KLH oligomerization state, molecular integrity and presence of contaminants by transmission electron microscopy (TEM) of negatively stained specimens at the start and throughout any purification procedure adds considerably to the validity of the data. As a first step in the purification of KLH, centrifugal pelleting has become standard. This differential centrifugation enables the high-molecular-mass hemocyanin to be separated from the other lower-molecular-mass hemolymph proteins, most of which remain in the supernatant. Centrifugation of cell-free hemolymph provides quantitative recovery of total hemocyanin, which can be suspended as a very high concentration KLH solution and is extremely convenient for further biochemical work; at this stage it should not be considered to be a highly purified protein, but see Swerdlow et al. [9]. The ratio of KLH1 to KLH2 varies considerably in the pelleted hemocyanin from individual animals. An average of approximately 1:2 for KLH1:KLH2 has been obtained from 325 animals [16]. In practice, it can be readily appreciated that the development of successful biochemical purification of intact KLH has been heavily dependent upon two techniques for monitoring the various separations: (i) by TEM and (ii) by crossed immunoelectrophoresis, be these achieved by ion exchange or gel filtration column chromatographic procedures. Early indications showed that animals held captive in saltwater aquaria for a period of months slowly lost most if not all of their KLH1. This enabled studies to be performed on a naturally occurring highly enriched population of KLH2 molecules, as in some animals KLH1 becomes undetectable. The health of such animals often appeared to be compromised and they do not feed well. The possibility exists that these animals are in severe negative protein balance and that they are utilizing the KLH1 to physiologically gain amino acids as well as the copper atoms.

Gel filtration chromatography of the mixed population of KLH1 and KLH2 oligomers yields a mass-dependent fractionation but little separation of the two KLH isoforms [6, 7, 17]. For both types of KLH the didecameric oligomer usually predominates. KLH1 also forms random didecamer clusters and KLH2 has a tendency to form elongated stacked-decamer oligomers/multimers, which are usually termed multidecamers. Gel filtration chromatography does not have the potential to discriminate between these higher oligomeric forms, and indeed under in vitro conditions inherent dynamic instability of the KLH2 didecamers leads to the formation of single decamers, in turn promoting a progressive increase in the number and length of the multidecamers during prolonged storage. When gel filtration chromatography is applied to the KLH2 taken from animals depleted with respect to KLH1, with the above caution in mind, it does nevertheless have the potential to produce a mass-dependent separation of the KLH2 multidecamers, didecamers and decamers [18].

A most important breakthrough occurred in 1995, when we gained some understanding of the confusing stability properties of total KLH in the presence of the ammonium molybdate solution used for the preparation of TEM specimens [19]. We found that at pH 7.0 the KLH2 multidecamers had a tendency to dissociate into decamers [7, 17] and that, when the pH was reduced further to the range of 5.7–5.9, the KLH2 dissociated into subunits, whereas the KLH1 (individual didecamers and didecamer clusters) remained intact. This provided us with the possibility to achieve separation of the two types of KLH by gel filtration chromatography in the presence of ammonium molybdate, with the KLH1 eluting first as 3 peaks (didecamer clusters, pairs of didecamers and didecamers) followed by a fourth peak containing the KLH2 subunit. This purification procedure was also found to be successful with total KLH reassociated from the commercially available subunit product Immucothel, following reassociation of both KLH isoforms in the presence of a high concentration of calcium and magnesium ions [16] (see below).

Herscowitz et al. [3] indicated that ion exchange chromatography on DEAE cellulose has the potential to fractionate total KLH, but at this early stage the two isoforms of KLH had not yet been defined. Indeed this alternative purification procedure, using ion exchange HPLC to separate KLH1 from KLH2 with both molecules in an intact

oligomeric state, has proven to be difficult to control and establish as a reproducible method [9, our unpubl. data]. Whilst it is possible to achieve a good separation of the KLH2 by HPLC, there is some carryover of the leading KLH2 peak into the following KLH1 peak. The adsorption of both KLHs, which are relatively fragile macromolecules, onto the ion exchange column matrix and release by elution with an increasing salt concentration could generate molecular instability. Rechromatography of the KLH1 through the HPLC column can produce higher purity, but this then becomes an extremely long procedure. Nevertheless, for bulk separations (i.e. gram quantitites) both ion exchange and gel filtration chromatography have considerable potential.

Molecular Mass Determinations with KLH

Although analytical ultracentrifugation has been widely used for the determination of the sedimentation coefficient (from sedimentation velocity analysis) and molecular weight (from sedimentation equilibrium analysis) of molluscan hemocyanins [20], this technique does suffer from a lack of sensitivity, particularly in any system where a range of similar oligomeric/multimeric states is present, together with some dynamic instability and/or aggregation of the molecules. Early indications from KLH showed the presence of multimeric forms [3], with predominant species sedimenting at 15S, 46S, 88S and 116S, but without any corresponding TEM images. Swerdlow et al. [9] established the values of 67S and 61S for the KLH1 and KLH2 decamers, respectively, and 100S and 105S for the KLH1 and KLH2 didecamers. These sedimentation values appear to be somewhat mutually contradictory, suggestive of the inherent limitation within the accuracy of this approach. For the KLH1 and KLH2 subunits, figures in the range of 11S to 13S were obtained [9]. After reassociation, the picture emerging from ultracentrifugation was even more confusing, indicating the limitations of this approach, particularly when no parallel TEM data on the samples were presented. On the other hand, if purified and stable oligomers are subjected to analytical ultracentrifugation, accurate sedimentation coefficient and molecular weight data are likely to be obtained. Mass determination obtained from unstained dark-field images of KLH1 and KLH2 (subunits and didecamers) by scanning transmission electron microscopy (STEM) [14] provided quantitative data which correlate well with the sedimentation coefficient values, and at the same time confirm the presence of several oligomeric forms. Light scattering has also been used for the study of hemocyanins and their subunits [21], and in combination STEM for assessment of the oligomerization states [22]. As with ultracentrifugation, light scattering is an indirect physical approach that has considerable limitations for the study of complex mixtures but is very useful for the study of solutions of highly purified monomeric species. Mass spectrometry has recently been introduced for the determination of accurate molecular mass, for both low- and high-molecular-mass proteins [23], but this technique also requires samples of high purity and stability; it is likely to be of considerable value for present and future studies on molluscan hemocyanins, subunit fragments and individual FUs [24]. Molecular mass determination of the subunit, single FU (usually between 45 and 50 kD) and FU multimers by SDS-PAGE has provided accurate values, which correlate well with values from STEM [8, 14]. From all these data it appears that the molecular mass of the KLH2 subunit is somewhat lower (360 kD) than that of the KLH1 subunit (390 kD), in spite of the presence of 8 FUs in each KLH subunit. Correspondingly, by STEM mass analysis, values of 8.2 and 7.5 MD were obtained for the KLH1 and KLH2 didecamers, respectively [14]. This mass difference has also been convincingly shown by independent methods [9].

FU Structure of KLH1 and KLH2

The recent availability of purified KLH1 and KLH2 has enabled considerable analytical progress to be made into the subunit organization of the two protein molecules. Gebauer et al. [8] presented the first major contribution within which the molecular analysis of KLH2, obtained from KLH1-depleted animals held in aquaria, was described. This work drew significantly upon limited proteolytic cleavage of the 360 kD subunit by bovine trypsin and *Staphylococcus aureus* V8 protease into FU fragments and into the individual FUs by bovine elastase, followed by their immunochemical analysis (see below). The molecular masses of the FUs and the multi-FU fragments were determined by SDS-PAGE together with their N-terminal amino acid sequences. Interpretation of these data led to the definition of a sequence of 7 different FUs *(abcdefg)* within the subunit. Comparison of the KLH2 subunit and FU amino acid N-terminal sequence data with that available from other molluscan hemocyanins revealed considerable similarity [8]. Supportive information was presented by Swerdlow et al. [9]. On the basis of copper analyses, these authors did, however, suggest that

KLH2 contains 8 FUs. A recent reassessment of our own data has now shown that under our isolation conditions, the FU *h* has been overlooked in KLH2 because of rapid degradation during the proteolysis with elastase [Gebauer et al., submitted]. Analysis of HPLC-purified proteolytic cleavage products from KLH1 [14] by SDS-PAGE and N-terminal sequence determination has now also provided a complete data set of the 8 KLH1 FUs, which differ structurally from those of KLH2.

Carbohydrates of KLH

Knowledge of the oligosaccharides of KLH is limited, but it is widely acknowledged that these sugar components are likely to be of prime significance for the antigenicity of the intact molecules, along with purified FUs or peptides that may be available from genetic engineering in the future. The carbohydrate content of total KLH was calculated to be approximately 4% of the molecular mass [25], and similar figures have been obtained for other molluscan hemocyanins [24]. A significantly higher carbohydrate figure, 9%, has been obtained for the vineyard snail *(Helix pomatia)* hemocyanin, which correlates with the higher molecular mass of approximately 55 kD usually obtained by SDS-PAGE of the individual *H. pomatia* hemocyanin FUs. By gas liquid chromatography, mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine were shown to be present in KLH1 and in addition to these 4 carbohydrates, fucose was also detected in KLH2 [Stoeva et al., submitted]. Three different lectins (concanavalin A [Con A] indicative of N-glycosides, peanut agglutinin [PNA] indicative of Gal(β1–3)-GalNAc as the core complex of O-linked glycosides and *Amaranthus caudatus* agglutinin [ACA] indicative of the α-anomer of Gal(β1–3)-GalNAc) were used to identify the saccharide components and carbohydrate-peptide linkages of KLH.

Using KLH1 multi-FU fragments and single FUs (see below), by immunoblotting Con A (which is specific for α-mannose, the main carbohydrate component of N-linked glycosides) was found to bind to FUs 1*ab*, 1*de*, 1*fg* and 1*h*, indicating the presence of N-linked glycosides. The lectin PNA did not bind to these KLH1 FU pairs and FU *h*, indicating the absence of O-linked glycosides. FU 1*c*, on the other hand, did not bind Con A, PNA or ACA, indicating the absence of carbohydrates.

For KLH2, by immunoblotting Con A was found to bind to all multi-FU fragments and single FUs 2*a*, 2*b*, 2*d*, 2*e*, 2*f*, 2*g*, and 2*h*, but not to FU 2*c*, indicating that this FU does not contain any N-glycosylated carbohydrate chains. This is supported by its polypeptide primary structure (see below). However, FU 2*c* was the only FU of KLH2 to bind PNA, and this FU did not bind the lectin ACA, indicating that the core complex of this O-glycoside must be the β-anomer of Gal(β1–3)-GalNAc. Lectins PNA and ACA did not bind to FUs 2*a*, 2*b*, 2*d*, 2*e*, 2*f*, 2*g* and 2*h*, indicating the lack of O-linked glycosides in all these FUs of KLH2.

The multiplicity of the monoantennary and diantennary molluscan hemocyanin N-linked oligosaccharide chains is indicated by the stuctural analysis of the α_D-hemocyanin of *H. pomatia* [26]. Although our molecular mass and sequence data indicate that the situation in KLH is less complex, it can be anticipated that it may be several years before the full structure of the KLH1 and KLH2 oligosaccharides will be available. Nevertheless, detection of the β-anomer of Gal (β1–3)-GalNAc supports the data of Wirguin et al. [27], who considered this to be the cross-reacting epitope of KLH essential for its efficacy as an immunotherapeutic agent for the treatment of bladder carcinoma. Concerning the biological role of this O-glycoside in KLH2 FU-*c* and its absence in KLH1 FU-*c*, one can only speculate. In this context it should be remembered that during prolonged captivity of the animals, KLH1 selectively disappears from the hemolymph, whereas KLH2 persists [7, 8]. It has been proposed that in this period, KLH1 is used as an amino acid source, whereas the remaining KLH2 maintains oxygen transport. These specific physiological roles could explain the need for two different hemocyanins within the same animal. The present data now suggest that the presence of the O-glycoside in KLH2-*c* may allow a specific receptor on the surface of the midgut gland cells to distinguish between the two hemocyanin isoforms.

Immunological Analysis of KLH

In view of the importance of immunoelectrophoresis and immunoblotting for the structural analysis of hemocyanins, including KLH, some further comment on these techniques is appropriate. The long-established immunoelectrophoresis of hemocyanins and other water-soluble proteins in agarose gel [3, 28, 29] using polyclonal antibodies has been an indispensable tool to provide the current understanding of the antigenic individuality of the two KLH isoforms, initially with respect to the subunit structure of KLH1 and KLH2 [6, 7] and then for the identification and linear sequence determination of the 8 FUs of the two subunits [8, 14]. Although formation of immu-

noprecipitation patterns requires much more polyclonal antibody than does immunoblotting, in the present context it is superior to the latter, because it allows direct estimation of the structural relationship of the applied proteins. To date, crossed immunoelectrophoresis is still the only method which allows visualization of the full set of different FUs in a molluscan hemocyanin. From all these experiments it is very clear that the 8 FUs of both KLH isoforms are very different structurally, which means that KLH represents a cocktail of 16 immunologically distinct FUs. Indeed, sequence analysis of *Octopus* hemocyanin [30] has revealed differences of about 50% in the primary structure of the different FUs of this molluscan hemocyanin. Figure 2 shows the immunoelectrophoretic analysis of elastase-cleaved KLH1 and KLH2 subunits, with definition of the 8 FUs *(abcdefgh)* for both isoforms.

Once characterized, the potential of monoclonal antibodies as reagents for the further immunological analysis within electrophoretic and blotting systems is considerable. They are also of value for the production of immune complexes in solution, from which epitope locational analysis of the native molecules can be performed by defining in TEM images the position on the molecules where the monoclonal antibody is binding [18, 31].

Molecular Genetics of KLH

In order to isolate cDNA clones coding for KLH1 and KLH2, cDNA expression libraries have been synthesized. For the construction of oligo(dT) and randomly primed libraries, RNA from several different tissues from *M. crenulata* (e.g. mantle and midgut gland) and different developmental stages of freshly captive animals were used. The screening of seven differently constructed cDNA libraries was performed with different monoclonal and polyclonal antibodies, mixed or alone, but no cDNA encoding KLH was isolated. However, parallel studies using the closely related mollusc *Haliotis tuberculata* yielded cDNA coding for hemocyanin [32] for both hemocyanin isoforms (HtH1 and HtH2) which could be isolated and sequenced. Furthermore, the analyses of the HtH1 gene elucidated the entire genomic organization of this hemocyanin [B. Lieb, B. Altenhein and J. Markl, to be published]. After having correlated all this information, genomic DNA from *M. crenulata* was isolated, and investigation of the KLH genes by PCR is currently in progress. One future goal is to express single FUs or the whole subunit in bacteria or eucaryotic cell lines.

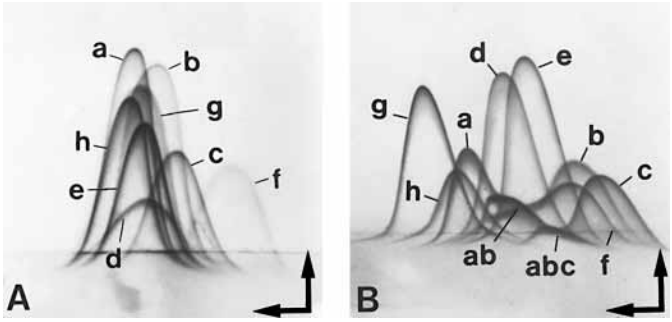


Fig. 2. The two-dimensional immunoelectrophoretic pattern of elastase-cleaved KLH1 (**A**) and KLH2 (**B**). For KLH1, the subunit has been completely cleaved into the 8 FUs *(abcdefgh)*. For KLH2, the *ab* FU dimer and the *abc* FU trimer are also present, along with the 8 individual FUs. Note that both FU panels do not cross-react immunologically. For a full interpretation of these data, see Gebauer et al. [8] and Söhngen et al. [14].

TEM Study of KLH

Despite the ready availablility of KLH and the large volume of early immunological data, study of this hemocyanin by TEM lagged considerably behind that of the hemocyanins from a number of other molluscs [33–36], but from 1990 TEM studies have contributed significantly to the structural characterization of the two isoforms of KLH, their oligomerization states and polymerization properties.

The Native Oligomers of KLH1 and KLH2

Whilst most samples of total KLH freshly isolated from individual animals contain a mixture of the two isoforms of KLH, the availability of some animals that were almost or totally depleted with respect to KLH1 has enabled an understanding of the oligomeric forms of KLH2, which consist of large and short multidecamers and didecamers. Starting from native mixtures of KLH1 and KLH2, dissociation of KLH2 in ammonium molybdate at pH 5.7, followed by gel filtration, yields a size-dependent separation of the KLH1 didecamer clusters from pairs or didecamers and single didecamers (fig. 3). The purified subunit of KLH2 elutes as the final material from the column. This KLH2 subunit is suitable for many biochemical and immunological studies and can be reassociated into decamers, didecamers, multidecamers and a tubular polymer if desired (see below). From purified KLH1 didecamers, it was possible to obtain a 15-Å three-dimensional reconstruction in air-dried negative stain +

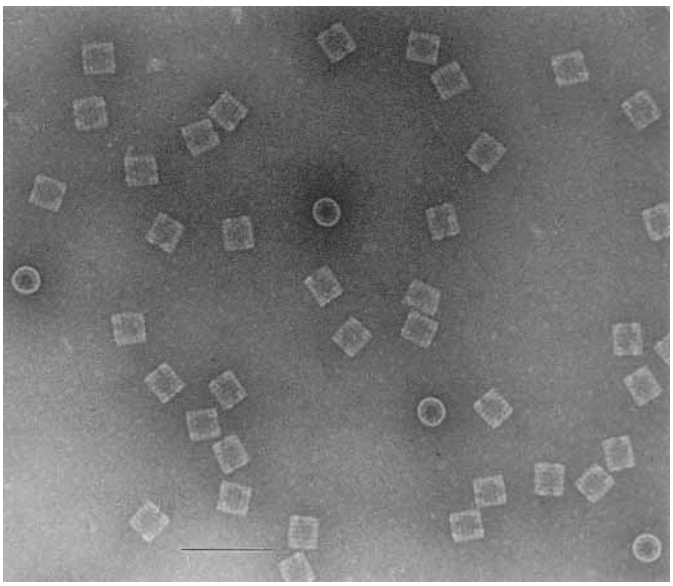
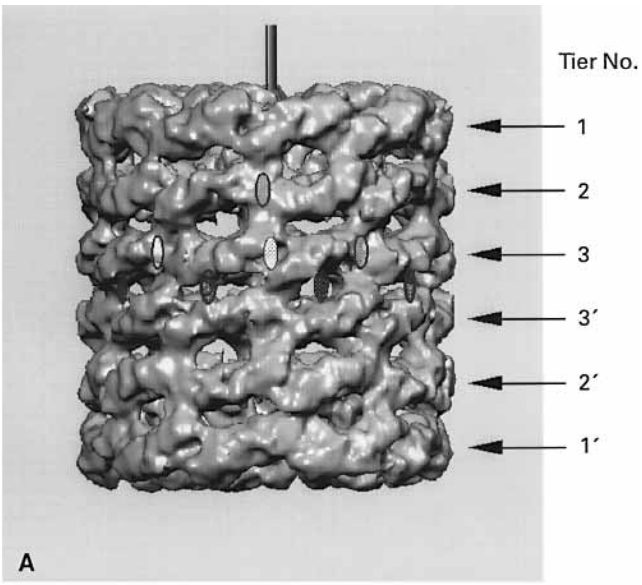


Fig. 3. Purified KLH1 didecamers obtained from total KLH by selective dissociation of the KLH2 and separation of the KLH1 by gel filtration chromatography [19]. The specimen was negatively stained with 5% ammonium molybdate containing 1% trehalose [37]. The scale bar indicates 100 nm.



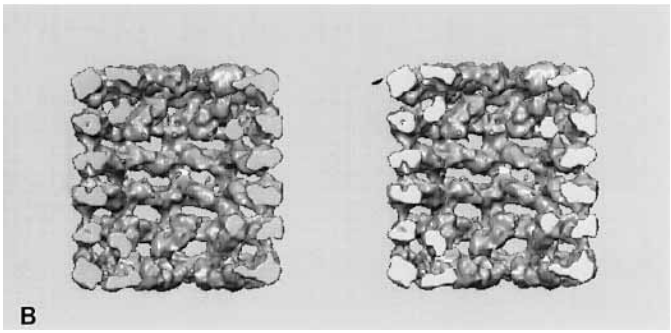


Fig. 4. The 15-Å resolution three-dimensional reconstruction of KLH1. **A** The outside view of the hollow cylindrical molecule, which has exact D5 point group symmetry. The three tiers of each of the two opposing decamers are indicated on the right-hand side: 1, 2, 3; 1′,2′,3′. **B** A stereo pair view of the inside of the KLH1 cylinder, cut open along the 5-fold axis. Note that each of the ovoid masses represents a 'morphological unit', consisting of 2 FUs. For a more complete account of these data, see Orlova et al. [38].

glucose at low temperature (~4 K) [38], from which many detailed features of the molecule emerge (fig. 4). However, it is not possible even at this high resolution to define the location of individual FUs, subunits or subunit pairs within the cylinder wall, but it is clear that the 10 masses forming each tier of the cylinder wall each represent an FU pair (often termed 'morphological units'). Moreover, the FU components of the internalized arc and collar components (at each end of the didecamer) have been identified as FUs *g* and *h*; yet their exact location within the arc and collar is not clear. Importantly, many symmetry features within the individual tiers of the didecamer wall are suggestive of a dyad symmetry within the FU pairs. These features are likely to be of importance for the future understanding of the manner in which subunit dimers are formed, which can in turn associate to form a closed pentameric ring (i.e. C5 symmetry) incorporating the 10 subunits of the decamer, subsequently creating the D5 didecamer. Whether or not the subunits within the subunit dimers are orientated in a parallel or antiparallel manner remains to be firmly established. Our earlier immunoelectron microscopy of KLH2 (see below) strongly implies that the subunit orientation within the decamer may be parallel, whereas the interpretation advanced by Orlova et al. [38] for the KLH1 didecamer suggests that the subunit orientation may be antiparallel, with complex interactions between the intricately associated subunits. Higher resolution TEM studies which permit the fitting of the known X-ray structure of FU *g* from *Octopus* hemocyanin [13] into the TEM structure may help to provide answers to these questions.

Immunoelectron Microscopy of KLH

Using monoclonal antibodies directed against epitopes on individual FUs (defined by immunoelectrophoresis), it has been possible to perform studies on the localization of FUs within IgG-linked immune complexes of KLH2 [18].

This approach carries with it some inherent limitations, in particular when steric hindrance of an epitope occurs within the overall quaternary structure. In theory, each decamer of KLH1 or KLH2 should have the possibility to bind a maximum of 10 IgG molecules. If, however, the position of the subunits within the molecule makes 5 of the epitopes inaccessible to the IgG, this fact may not be easily assessed. The use of purified decamers of KLH1 and KLH2 instead of didecamers could help to avoid anomalies due to this steric hindrance.

Whilst the use of *monovalent* Fab′ fragments provides the possibility for higher-resolution epitope analysis than does use of *bivalent* IgG, the mass difference between a single Fab′ fragment (approx. 50 kD) and a KLH decamer (approx 4 MD) is so great that in practice it is difficult to detect the bound Fab′, although one might expect that, if the maximum of 10 Fab′ fragments were attached per 4-MD decamer, this would generate a characteristic change to the image of individual decamers and didecamers. To date, Fab′ labeling has not been convincingly detectable for KLH, although others have achieved considerable success with other hemocyanins [39] but using polyclonal Fab′ fragments.

Experimental Manipulation of the Oligomeric States of KLH in vitro

Exactly why the didecamer is the predominant oligomeric form of both KLH1 and KLH2, in vivo and following isolation from hemolymph, is not understood. In all probability this relates to the asymmetric nature of the decamer, with the collar region at one edge of the ring-like molecule. The hemocyanin of Cephalopoda (e.g. octopus and squid) exists as stable decamers, with a central (symmetrical) collar. In addition, the instability of purified KLH2 didecamers, with the formation of single decamers and multidecamers (containing a nucleating didecamer, with decamers added at one or both sides) are further aspects that require explanation. In an attempt to answer some of these questions, we have established procedures for the production of stable decamers from KLH1 and KLH2, which can then be used for a range of different experimental approaches.

For KLH1, we have found that by simply increasing the pH of the Tris-saline stabilizing buffer to pH 8.5, the didecamers split into stable decamers [14]. This dissociation can be reversed by dialysis against pH 7.4 stabilizing buffer (with 5 m*M* calcium and magnesium chloride), and dialysis against pH 7.4 stabilizing buffer containing 100 m*M* calcium and magnesium chloride generates multidecamers. It should be noted that KLH1 multidecamers have not been detected in purified KLH1 samples obtained directly from animals; they can, however, also be produced by reassociation of the KLH1 subunit, under these high divalent cation conditions (see below). No nucleating didecamer can be defined within these KLH1 multidecamers and there is a structural difference between these and the KLH2 multidecamers, expressed by the varying negative stain distribution along their hollow centers. This is surprising because to date no convincingly defined structural difference has been detected between didecamers of KLH1 and KLH2, by comparison of computer-processed TEM images. On the other hand, both KLH isoforms consist of a different subset of FUs, and the two subunits are unable to coassemble [14].

For KLH2, the production of decamers has proven to be slightly more difficult, as simple adjustment of the stabilizing buffer pH had no effect. However, overnight dialysis against Tris buffer at pH 7.5 (i.e. in the absence of NaCl, $CaCl_2$ and $MgCl_2$) has been found to split purified samples of KLH2 (containing a mixture of didecamers and multidecamers) into a highly enriched population of decamers, but with some instability, indicated by breaking of some decamers. Further dialysis of these KLH2 decamers against the usual KLH stabilizing buffer causes didecamers to form again. Very significantly, when dialysis is performed against the stabilizing buffer containing 100 m*M* calcium and magnesium, greatly elongated multidecamers were formed, often with more than one nucleating didecamer, but sometimes with no didecamer present.

This experimental manipulation of the oligomeric states of KLH1 and KLH2 has provided data that correlate well with the known oligomeric forms obtained from total KLH samples and extends our understanding of multimer formation by the two decameric forms. It can be concluded that both isoforms of KLH exhibit characteristic oligomerization features, be these produced naturally or experimentally.

Subunit Reassociation Studies

The production of molluscan hemocyanin subunits by dialysis of the intact oligomer(s) against a high pH buffer, with or without the presence of EDTA, is long-established. Study of the hemocyanin dissociation intermediates has not been easily achieved. However, during the reverse process, subunit reassociation, this assembly process can be more readily monitored by electron microscopy and other physical techniques [21]. Rather than the early stages of subunit reassociation of KLH1 and KLH2, we have been concerned with the study of the stable end

products. This work has been performed with purified subunit material produced from total KLH samples and also from commercially available Immucothel®. Initially, in our studies we concentrated upon rapid (2 days) reassociation in the presence of 100 m*M* calcium and magnesium ions [15, 16] and more recently we have studied the slower (10 days) reassociation in the presence of 10 m*M* calcium and magnesium [40].

At the high divalent cation concentration, it was found that KLH1 reassociates to form a mixture of oligomeric forms and a polymeric tubular form. Among the oligomers the didecamer predominates during the initial reassociation of the mixed KLH1 and KLH2 subunits in Immucothel®, but following high pH dissociation and a repeated reassociation, multidecamers are also present. In the presence of ammonium molybdate and polyethylene glycol, the KLH1 tubules have the potential to convert directly into multidecamers [16].

The KLH2 subunit reassociates in the presence of stabilizing buffer containing 100 m*M* calcium and magnesium to form a mixture of multidecamers (sometimes of considerable length) and a right-handed helical/tubular polymer that is somewhat different from that produced by the KLH1 subunit under these ionic conditions. As with KLH1, the diameter of the KLH2 tubular polymer is less than that of the multidecamers. Electron-optical images of the multidecamers and tubular polymers of KLH1 and KLH2 each exhibit a different and characteristic Fourier transform [15]. Biochemical analysis of such samples demonstrated that in both forms the subunit is intact; thus, these tubular polymers do not contain proteolytically cleaved subunits.

Reassociation of both KLH1 and KLH2 subunits in the presence of 10 m*M* calcium and magnesium is much slower and slightly less complete than with 100 m*M* concentrations of these cations. The oligomeric forms of both hemocyanins then predominate over the tubular polymers, indicative of association conditions that are closer to those encountered under physiological conditions [40].

Following controlled proteolytic cleavage of KLH1 and KLH2 with the V8 protease from *Staphylococcus aureus* we have found that subunit fragments also possess the capacity to reassociate or to create helical tubular polymers [41], but with no decamers, didecamers or higher multidecameric oligomers. To date it has not been possible to produce proteolytically a KLH fragment containing only the six wall FUs. The future availability of such material presents further interesting possibilities for reassociation studies, as will cloned subunits and multi-FU fragments of KLH1 and KLH2, both as pure proteins and following biosynthetic or chemically produced glycosylation.

Conclusions

A survey of the currently available biochemical, immunochemical and ultrastructual data on KLH has been presented, with the intention of providing a broad scientific background for the various oncological uses of KLH, as discussed by other contributors to this issue of *European Urology*.

Acknowledgements

We thank our colleagues Wolfgang Gebauer and Bernhard Lieb for useful information, Käthe Rehbinder for the drawing and the Deutsche Forschungsgemeinschaft (grant 843/4 to J.M.) for continuing support for our ongoing studies on KLH. Our TEM studies are supported by the Stiftung Innovation von Rheinland Pfalz (No. GZ. 8312-386261/281). We also acknowledge the constant interest and support of the Biosyn Company.

References

1 Curtis JE, Hersh EM, Harris JE, McBride C, Freireich EJ: The human primary immune response to keyhole limpet hemocyanin: Interrelationships of delayed hypersensitivity, antibody response and in vitro blast transformation. Clin Exp Immunol 1970;6:473–491.

2 Curtis JE, Hersh EM, Butler WT, Rossen RD: Antigen dose in human immune response, dose response relationship in the human immunoresponse to keyhole limpet hemocyanin. J Lab Clin Med 1971;78:61–69.

3 Herscowitz HB, Harold WW, Stravitzky AB: Immunochemical and immunogenetics properties of a purified keyhole limpet hemocyanin. Immunology 1972;22:51–61.

4 Weigle WO: Immunochemical properties of hemocyanin. Immunochemistry 1964;1:295–302.

5 Senozan NM, Landrum J, Bonaventura J, Bonaventura C. Hemocyanin of the giant keyhole limpet, *Megathura crenulata*; in Lamy J, Lamy J (eds): Invertebrate Oxygen-Binding Proteins. New York, Dekker, 1981, pp 703–717.

6 Savel-Niemann A, Wegener-Strake A, Markl J: Keyhole limpet hemocyanin: On the structure of a widely used immunological tool; in Preaux G, Lontie R (eds): Invertebrate Dioxygen Carriers. Leuven, Leuven University Press, 1990, pp 351–356.

7 Markl J, Savel-Niemann A, Wegener-Strake A, Süling M, Schneider A, Gebauer W, Harris R: The role of two distinct subunit types in the architecture of keyhole limpet hemocyanin (KLH). Naturwissenschaften 1991;78:512–514.

8 Gebauer W, Harris R, Heid H, Süling M, Hillenbrand R, Söhngen S, Wegener-Strake A, Markl J: Quaternary structure, subunits and domain patterns of two discrete forms of keyhole limpet hemocyanin: KLH 1 and KLH 2. Zoology 1994;98:51–68.
9 Swerdlow RD, Ebert RF, Lee P, Bonaventura C, Miller KI: Keyhole limpet hemocyanin: Structural and functional characterization of two different subunits and multimers. Comp Biochem Physiol B 1996;113:537–548.
10 Sminia T: Haemocyanin-producing cells in gastropod molluscs; in Bannister JV (ed): Structure and Function of Haemocyanin. Berlin, Springer, 1977, pp 279–288.
11 Albrecht U: Zum Biosyntheseort des Hämocyanins bei Schnecken: Ultrastruktur der Porenzellen von *Megathura* im Vergleich zu *Haliotis* und *Lymnaea*; PhD thesis, Institut für Zoologie, Universität Mainz, 1996.
12 Salvato B, Beltramini M: Hemocyanin molecular architecture: Structure and reactivity of the binuclear copper active site. Life Chem Rep 1990;8:1–47.
13 Cuff M, Miller KI, van Holde KE, Hendrickson WA: Crystal structure of a functional unit from octopus hemocyanin. J Mol Biol 1998; 278:855–870.
14 Söhngen SM, Stahlmann A, Harris JR, Muller SA, Engel A, Markl J: Mass determination, subunit organization and control of oligomerization states of keyhole limpet hemocyanin (KLH). Eur J Biochem 1997;248:602–614.
15 Harris JR, Gebauer W, Söhngen SM, Nermut MV, Markl J: Keyhole limpet hemocyanin (KLH). II. Characteristic reassociation properties of purified KLH1 and KLH2. Micron 1997;28:43–56.
16 Harris JR, Gebauer W, Guderian FUM, Markl J: Keyhole limpet hemocyanin (KLH). I. Reassociation from Immucothel® followed by separation of KLH1 and KLH2. Micron 1997;28: 31–41.
17 Harris R, Cejka Z, Wegener-Strake A, Gebauer W, Markl J: Two-dimensional crystallization, transmission electron microscopy and image processing of keyhole limpet hemocyanin (KLH). Micron Microsc Acta 1992;23:287–301.
18 Harris JR, Gebauer W, Markl J: Immunoelectron microscopy of hemocyanin from the keyhole limpet *(Megathura crenulata):* A parallel subunit model. J Struct Biol 1993;111:96–104.
19 Harris JR, Gebauer W, Söhngen SM, Markl J: Keyhole limpet haemocyanin (KLH): Purification of intact KLH1 through selective dissociation of KLH2. Micron 1995;26:201–212.
20 Van Holde KE, Miller KI: Hemocyanins. Adv Protein Chem 1995:1–81.
21 van Holde KE, Miller K, Schabtach E, Libertini L: Assembly of *Octopus dofleini* hemocyanin: A study of the kinetic by sedimentation, light scattering and electron microscopy. J Mol Biol 1991;217:307–321.
22 Hamilton MG, Herskovits TT, Furcinitti PS, Wall JS: Scanning transmission electron microscopic study of molluscan hemocyanins in various aggregation states: Comparison with light scattering molecular weights. J Ultrastruct Mol Struct Res 1989;102:221–228.
23 Martin PD, Kuchumov AR, Green BN, Oliver RWA, Braswell EH, Wall JS, Vinogradov SN: Mass spectrometric composition and molecular mass of *Lumbricus terrestris* hemoglobin: A refined model of its quaternary structure. J Mol Biol 1996;255:154–169.
24 Stoeva S, Idakieva K, Rachev R, Voelter W, Genov N: Amino terminal oxygen binding functional unit of the *Rapana thomasiana* grosse (gastropod) hemocyanin: Carbohydrate content, monosaccharide composition and amino acid sequence studies. Comp Biochem Physiol B 1997;117:101–107.
25 van Kuik JA, Kamerling JP, Vliegenthart JFG: Carbohydrate analysis of hemocyanins; in Preaux G, Lontie R (eds): Invertebrate Oxygen Carriers. Leuven, Leuven University Press, 1990 pp 157–163.
26 Lommerse JP, Thomas Oates JE, Gielens C, Preaux G, Kamerling JP, Vliegenthart JF: Primary structure of 21 novel monoantennary and diantennary N-linked carbohydrate chains from alphaD-hemocyanin of *Helix pomatia.* Eur J Biochem 1997;249:195–222.
27 Wirguin I, Suturkova Milosevic L, Briani C, Latov N: Keyhole limpet hemocyanin contains Gal(beta 1–3)-GalNAc determinants that are cross-reactive with the T antigen. Cancer Immunol Immunother 1995;40:307–310.
28 Kroll J: Crossed-line immunoelectrophoresis. Scand J Immunol Suppl 1973;1:79–81.
29 Weeke B: Crossed immunoelectrophoresis. Scand J Immunol Suppl 1973;1:47–56.
30 Miller KI, Cuff ME, Lang WF, Varga-Weisz P, Field KG, van Holde KE: Sequence of the *Octopus dofleini* hemocyanin subunit: Structural and evolutionary implications. J Mol Biol 1998;278:827–842.
31 Harris JR: Immunonegative staining: Epitope localization on macromolecules. Methods 1996;10:234–246.
32 Lieb B, Altenhein B, Lehnert R, Gebauer W, Markl J: Subunit organization of the abalone *Haliotis tuberculata* hemocyanin type 2 (HtH2), and the cDNA sequence coding for its functional units d, e, f, g and h. Eur J Biochem 1999;265:134–144.
33 Fernandez-Moran H, Van Bruggen EFJ, Ohtsuki M: Macromolecular organization of hemocyanins and apohemocyanins as revealed by electron microscopy. J Mol Biol 1966;16:191–207.
34 Van Bruggen EFJ, Wiebenga EH, Gruber M: Structure and properties of hemocyanins. II. Electron micrographs of the hemocyanin of *Sepia officinalis, Octopus vulgaris* and *Cancer pagurus.* J Mol Biol 1962;4:8–9.
35 Van Bruggen EFJ, Wiebenga EH, Gruber M: Structure and properties of hemocyanins. I. Electron micrographs of hemocyanin and apohemocyanin from *Helix pomatia* at different pH values. J Mol Biol 1962;4:1–7.
36 Mellema JE, Klug A: Quaternary structure of gastropod haemocyanin. Nature 1972;239: 146–150.
37 Harris JR, Gebauer W, Markl J: Keyhole limpet haemocyanin: Negative staining in the presence of trehalose. Micron 1995;26:25–33.
38 Orlova EV, Dube P, Harris JR, Beckman E, Zemlin F, Markl J, Vanheel M: Structure of keyhole limpet hemocyanin type 1 (klh1) at 15 angstrom resolution by electron cryomicroscopy and angular reconstitution. J Mol Biol 1997; 271:417–437.
39 Lamy J, You V, Taveau J-C, Boisset N, Lamy JN: Intramolecular localization of the functional units of *Sepia officinalis* hemocyanin by immunoelectron microscopy. J Mol Biol 1998; 284:1051–1074.
40 Harris JR, Gebauer W, Adrian M, Markl J: Keyhole limpet hemocyanin (KLH): Slow in vitro reassociation of KLH1 and KLH2 from Immucothel. Micron 1998;29:329–339.
41 Gebauer W, Harris JR: Controlled cleavage of KLH1 and KLH2 by the V8 protase from *Staphylococcus aureus.* Reassociation, electrophoretic and transmission electron microscopy study of peptide fragments. Eur J Biochem 1999;262:166–175.