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HYDROGEN EXCHANGE IN HYDRATED FILMS OF PROTEINS. APPLICATION TO THE *E. COLI LAC* REPRESSOR CORE

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An original easy method of hydrogen to deuterium exchange in hydrated films of proteins, followed by infrared absorption measurements, is described and applied to films of the *E. coli lac* repressor core, in order to examine the effect of isopropyl-β-D-thiogalactoside (IPTG) binding. An estimation of about 25% α helical structure in this protein fragment is deduced from the exchange curve. The binding of IPTG to the core does not affect the exchange curve within the experimental error limits.

1. Introduction

The hydrogen exchange rates of peptide NH groups are known to be very sensitive to protein conformations. They depend upon hydrogen bonding in α helix or β sheets, and upon hydrophobic and steric environment of the labile protons in the molecule [1–3]. Some difficulties in hydrogen exchange techniques, when measured by infrared absorption spectroscopy on solutions arise from the necessity to dissolve quickly (which is often no possible) the lyophilized protein in D_2O, and to introduce this solution into a cell which is only a few microns thick in a very short period of time. The very fast exchange of some protons at neutral pH, with half-life of the order of one second [4,5] does not allow extrapolation of the exchange curve to zero time. Thus the protein absorbance in the initial undeuterated state is not known without indirect estimation. A similar problem exists in determining the total number of labelled protons at zero time in tritium-hydrogen exchange experiments which are measured by gel filtration and radioactivity counting [2,6–8]. Furthermore, in this latter exchange-out method, very slow exchanging hydrogens are very difficult to label except if the incubation time is increased or if the conditions of incubation are more drastic (for example heating the solution).

We describe here a simple method to follow the hydrogen-deuterium exchange of peptide N-H groups by infrared absorption measurements of the amide II absorbance at 1550 cm^{-1}, in hydrated films of proteins, when exposed to heavy water D_2O vapour. This method allows one to determine the initial point of the exchange curve, and does not require any lyophilization of the protein, as well it avoids the difficulties of infrared spectroscopy on dilute solutions. In this work we describe how we have used these techniques to study the *lac* repressor core and its interaction with an inducer.

Under certain conditions proteolytic cleavage of the *lac* repressor occurs between residues 50 and 60 yielding two fragments: the N-terminal headpiece, which contains the major portion (if not all) of the DNA binding site, and a tetrameric core with full inducer binding activity [9,10]. We were interested in studying the effect of an inducer, in this work isopropyl-β-D-thiogalactoside (IPTG), on this core, in order to obtain further information on its mechanical action. This core has the advantage of a greater solubility than the intact protein, allowing the formation of concentrated homogeneous films of the protein and their study by infrared absorption spectroscopy.

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2. Materials and methods

E. coli lac repressor from strain BMH 493 (a generous gift of Dr. Beyreuther and Dr. Müller-Hill) was purified by ammonium sulfate fractionation and column chromatography on phosphocellulose as described by Müller-Hill et al. [11]. Proteolysis was performed as described by Geisler and Weber [12] using clostripain (Worthington). The core preparation was analyzed on SDS polyacrylamide gel and run as a single band corresponding to molecular weight about 32 000 accounting for residues 60 to 360.

Films of repressor core were deposited on calcium fluoride (CaF_2) IR windows by gentle drying at ambient temperature and humidity. 1 ml of a solution 4.7×10^{-5} M core (i.e. 1.5 mg/ml) as measured by UV spectroscopy (using $\epsilon_{280} = 16\,600$ per protomer), in 10^{-3} M phosphate buffer, pH 7.3, 10^{-4} dithioerythritol was used. In some experiments the sample was made 10^{-4} M IPTG, to insure saturation of the inducer binding site in the core.

The film on the window was then put into a cell, closed with a second window, and a humidity of 98% R.H. was achieved by placing 1 ml of a saturated salt solution of lead nitrate $Pb(NO_3)_2$ in a teflon cylinder in the bottom of the cell. This teflon cylinder could be easily and quickly exchanged without opening the cell by having a new cylinder displacing the first one.

The cell was placed in an IR 12 infrared spectrophotometer. A similar cell, without any film was placed in the reference beam. The cells were thermostated by water circulation at 29 ± 2°C as measured with a thermistor in close contact with the window supporting the sample. The first spectrum from 1000 to 4000 cm^{-1}, in the initial hydrogenated state, was run when equilibrium of temperature and water content (as measured using the 3400 cm^{-1} band of water) had been obtained (usually after 1 or 2 hours). At zero time, the teflon cylinder containing the salt saturated water solution was exchanged for saturated heavy water solution previously equilibrated to the same temperature. The absorbance of the film was recorded at fixed frequency of 1550 cm^{-1} (Amide II band) as a function of time. From time to time spectra were run from 1500 to 1660 cm^{-1} during the exchange experiment. A complete spectrum from 1000 to 4000 cm^{-1} was run in the final deuterated state.

The absorption measurements have been corrected for non linearity of the refernce comb and the absorbance uncertainty was only 0.003 absorbance units. The core films were controlled to present rather good constant thickness, without any holes in the beam. The spectral slit width used was 5 cm^{-1} allowing negligible noise.

The film, containing about 1.5 mg of protein, contains about an equivalent quantity of water, as shown by the water absorbance at 3400 cm^{-1}. On the other hand, the internal volume of the cell is about 10 ml which contains at 29°C about 0.3 mg water vapour at 98% R.H. Thus the total initial amount of light water is negligible in terms of the quantity of heavy water (~1 g) which is placed in the cell with the teflon cylinder.

The exchange from light to heavy water in the vapour phase is very fast (halftime about 20 s) as measured by the disappearance of the 1560 cm^{-1} water vapour absorption in control experiments. The half time of the exchange between the absorbed water molecule in the thin protein film and the heavy water from the vapour phase, is between 1 and 10 minutes as seen by disappearance of the 3400 cm^{-1} water absorption band in control experiments. This is also the order of magnitude of the initially observed peptide hydrogen exchange rate at the beginning of the experiment, as calculated from the exchange curves (see results). The rate constant for the exchange of the fastest hydrogens, in the case of random polypeptides is theoretically given by [2,5,13]:

$$k_0 = 50(10^{-pH} + 10^{pH-6.0}) \times 10^{0.05(\theta-20)}\ min^{-1}. \quad (1)$$

This gives with $\theta = 29$°C and pH 7.3 a values of $k_0 = 2.9 \times 10^3\ min^{-1}$, i.e. a halftime life of 2.5×10^{-4} min. Thus the exchange of this class of proton cannot be observed by this method, since it is faster than the exchange of the water molecules between the film and the vapour phase.

3. Results and discussion

Fig. 1 shows the exchange curves for the repressor core, in the presence and in the absence of IPTG. These curves represent the absorbance ratio $A(t)/A(0)$ at 1550 cm^{-1} as a function of time where $A(t)$ is the absorbance and $A(0)$ the initial absorbance. This ratio is a

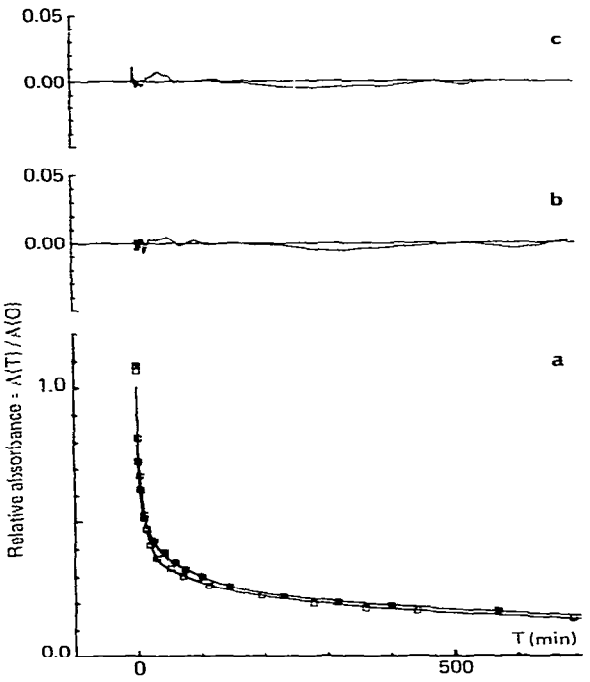

Fig. 1. a) Hydrogen exchange data for free (▫) and for IPTG saturated (▪) *E. coli lac* repressor core. Relative absorbance $A(t)/A(0)$, which is the ratio of the absorbance at 1550 cm^{-1} at time T, $A(T)$, to the initial absorbance, $A(0)$, versus time T. For figure clarity we show only a few of the experimental points we used for calculations. Full lines represent the curves calculated from parameters in table 1 (four-class analysis). b) and c). × 5 magnified deviations of experimental points from calculated curves, b) for free and c) for IPTG saturated repressor core.

measurement of the $(n_0 - n_t)/n_0$ fraction (2), n_0 being the total number of peptide NH hydrogens in the initial state, and n_t the number of these hydrogen atoms exchanged for deuterium at time t.

We have analyzed the exchange curves as a sum of first order terms (2):

$$\frac{n_0 - n_t}{n_0} = \sum_{i=1}^{N} \frac{n_i}{n_0} \exp(-k_i t), \qquad (2)$$

where k_i is the exchange rate of a class containing n_i hydorgen atoms, and N the number of such classes. We used a computer successive substraction method for this calculation: the weighted least square best fit of the last experimental points, on a semi logarithmic scale, gives the size n_1/n_0 and the exchange rate k_1 for the slowest hydrogen class. Then after substraction of this class from the experimental data, we obtained sizes and rates for the N classes by iterative techniques of the same calculation.

Since Laiker and Prinz [14] have shown that a same exchange curve could be fitted by several computed curves, with different numbers N of classes, we also used the more sophisticated Provencher's eigenfunctions expansion method [15] for the same calculations. This method permits objective determination of the number N of classes. The results given by Provencher's "discrete" calculation program agree very well, within the errors limits, with the four-class analysis of the successive substractions method, which is valid here because the well separated exchange rate values of the different hydrogen classes.

Exchange curves are shown on fig. 1 and results of the calculation are shown in table 1. The calculated values for the half-times of the two fastest classes in the four-class analysis are lower than 10 minutes, i.e. in the order of the D_2O penetration half-time in the film. For this reason we think that the separation of these two fastest classes is an artifact. Control experiments shows that the sizes of these two classes are not reproducible, whereas the sum of their sizes is reproducible.

So, we will consider them thereafter to form only one class with an unknown half-time shorter than 10 minutes.

It is clear from table 1 that the experimental data for free and IPTG saturated repressor core are best fit by very similar parameters within the experimental error, when only three reproducible classes are considered, i.e. that binding of IPTG to the repressor core does not affect the exchange law.

As often made by several authors [2,16], we can try to relate the size of the various classes to the α helical, β sheet and random part fractions of the protein. However, it is necessary to make clear that this relation is only tentative since it is well known that the secondary structure is only one among the elements which determine the exchange rate of a residue [3,17,18].

Analysis of the circular dichroism spectrum of the *lac* repressor core by Chou et al. [19] gave a content of

Table 1

Class i	Free *lac* repressor core				*lac* repressor core + IPTG			
	Size n_i/n_0	Protons number n_i	Half-time [b] (min)	Exchange rate (min^{-1})	Size n_i/n_0	Protons number n_i	Half-time [b] (min)	Exchange rate (min^{-1})
Very slow $i = 1$	23% ± 3%	62 – 80	650 – 1400 [c]	$0.5 \times 10^{-3} - 1.1 \times 10^{-3}$	24% ± 2%	68 – 80	800 – 1400 [c]	$0.5 \times 10^{-3} - 0.9 \times 10^{-3}$
	(0.23)	(71)	(940)	(0.74×10^{-3})	(0.24)	(74)	(1020)	(0.68×10^{-3})
Slow $i = 2$	16% ± 4%	37 – 62	75 ± 15	$0.8 \times 10^{-2} - 1.2 \times 10^{-2}$	22% ± 3%	58 – 77	60 ± 10	$1.0 \times 10^{-2} - 1.4 \times 10^{-2}$
	(0.16)	(49)	(73)	(0.94×10^{-2})	(0.22)	(68)	(62)	(1.12×10^{-2})
Fast $i = 3$	61% ± 7%	165– 210	<10	$>7 \times 10^{-2}$	54% ± 5%	150 – 180	<10	$<7 \times 10^{-2}$
	(0.61)	(188)		(0.14)	(0.54)	(166)		(0.20)
or								
$i = 3$	(0.38) [d]		(7.0) [d]		(0.18) [d]		(7.9) [d]	
$i = 4$	(0.23) [d]		(1.1) [d]		(0.36) [d]		(1.2) [d]	

a) Values in parentheses are those calculated for the best fit. The underlined values give range of uncertainty.

b) The half-time $t^{1/2}$ is related to the exchange rate k by the relationship: $t^{1/2} = 0.693/k$.

c) The presence in this class of some more slowly exchanging hydrogens cannot be excluded since the measurements have been made during 700 min. only.

d) These values correspond to a four class analysis, fitting the experimental curve to 0.01 relative absorbance units, but with lack of experimental reproducibility (see text).

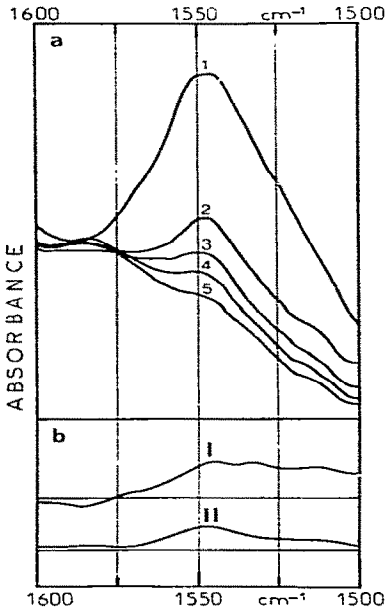

Fig. 2. a) Infrared absorption spectrum of the *E. coli lac* repressor core (saturated with IPTG). 1 in the initial, hydrogenated state, 2 after 20 min exchange, 3 after 50 min, 4 after 130 min, 5 after 1330 min. b) Difference spectrum, I between 20 and 50 min exchange, accounting mainly for the second, slow class, II between 130 and 1330 min, accounting mainly for the first, very slow class. Free repressor core spectra are not different from the above ones.

α-helix between 16 and 28%. Using a similar analysis but with an other set of reference spectra, we calculated an α helical content of 28% (Szabo and Maurizot, to be published). The method of conformational prediction of Chou and Fasman [20] indicates a value of 29% for α-helical content when applied to the *lac* repressor core [19]. All these values are in agreement with our results, assuming that the very slow class of protons corresponds to the α helix regions of the protein.

To confirm this point, the difference spectrum between the protein in its maximum deuterated state (after 1330 min) and protein at the end of the previous class (130 min) was drawn in the amide II region between 1500 and 1600 cm^{-1}. It is in good agreement with known spectra of α-helical proteins in H_2O [21–23] with maxima at about 1545 and 1515 cm^{-1} further supporting our assignment.

For the second slow class and for the third class the situation is not so clear, since the IR spectrum of the random regions of proteins is not well known and is probably different for different proteins. The difference spectrum of amide II band, between 20 and 50 minutes is largely accounted for by the second class and shows maxima at about 1545 and 1535 cm^{-1} which may be due to the H bonded NH groups in β-sheet [21–24]. However it is possible that this class contains some solvent-exposed α-helical regions and buried random regions of the protein [2,3,8].

Similarly the third, fast class cannot be attributed only to random parts of the protein and probably includes some H bonded exposed hydrogens of the peptice units.

4. Conclusions

Our results show that the hydrogen to deuterium exchange methods can be applied to hydrated films in order to study the conformations of proteins. The analysis of the exchange curve in terms of classes can give an approximate estimate of the α helical content of the protein.

In the case of *lac* repressor core we found an α helical content of approximately 25% in good agreement with other analyses and predictions [19,25].

Our results show that the exchange curve does not vary upon binding the inducer IPTG. This indicates that the inducer binding does not cause a large conformational change in the core part of the *lac* repressor. These results are in agreement with those of Ramstein et al. [26] obtained by the tritium hydrogen exchange using the tritium tracer method. They support the idea that the effect of the inducer binding to the core is mainly related to the interaction between the "headpiece" and the core of the *lac* repressor.

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References

[1] K. Linderstrom-Lang, C.R. Trav. Lab. Carlsberg 15 (1964) 7.
[2] A. Hvidt and S.O. Nielsen, in: Advances in protein chemistry, eds. C.B. Anfinsen Jr., M.L. Anson, J.T. Edsall and F.M. Richards (Acad. Press, New-York, 1966) p. 287.
[3] S.W. Englander, N.W. Downer and H. Teitelbaum, Ann. Rev. Biochemistry 41 (1972) 903.
[4] E.R. Blout, C. de Loze and A. Asadourian, J. Am. Chem. Soc. 63 (1961) 1895.
[5] S.W. Englander and A. Poulsen, Biopolymers 7 (1969) 379.
[6] S.W. Englander and C. Mauel, J. Biol. Chem. 247 (1972) 2387.
[7] S.W. Englander and R. Staley, J. Mol. Biol. 45 (1969) 277.
[8] S.W. Englander and J.J. Englander, in: Methods in enzymology, XXVI, part C, eds. C.H.W' Hirs and S.N. Timasheff (Acad. Press, New-York, 1972) p. 406.
[9] N. Geisler and K. Weber, FEBS Letters 87 (1978) 215.
[10] T. Platt, J.G. Files and K. Weber, J. Biol. Chem. 248 (1973) 110.
[11] B. Muller-Hill, K.T. Beyreuther and W. Gilbert, in: Methods in enzymology, XXI, part D, eds. L. Grossman and K. Moldave (Acad. Press, New-York, 1974) p. 483.
[12] N. Geisler and K. Weber, Biochemistry 16 (1977) 938.
[13] A. Hvidt and K. Wallevik, J. Biol. Chem. 247 (1972) 1530.
[14] S.L. Laiken and M.P. Prinz, Biochemistry 9 (1970) 1547.
[15] S.W. Provencher, J. Chem. Phys. 64 (1976) 2772.
[16] M. Nakanishi and M. Tsuboi, Biochim. Biophys. Acta 434 (1976) 365.
[17] B.D. Hilton and C.K. Woodward, Biochemistry 7 (1978) 3325.
[18] G. Wagner and K. Wüthrich, J. Mol. Biol. 134 (1979) 75.
[19] P.Y. Chou, A.J. Adler and G.D. Fasman, J. Mol. Biol. 96 (1975) 29.
[20] P.Y. Chou and G.D. Fasman, Biochemistry 13 (1974) 222.
[21] S.N. Timasheff and H. Susi, J. Biol. Chem. 241 (1966) 249.
[22] H. Susi, S.N. Timasheff and L. Stevens, J. Biol. Chem. 242 (1967) 5460.
[23] H' Susi, in: Methods in enzymology, vol. XXVI, part C, eds. C.H.W. Hirs and S.N. Timasheff (Acad. Press, New-York, 1972) p. 455.
[24] T. Miyazawa and E.R. Blout, J. Am. Chem. Soc. 83 (1961) 712.
[25] S. Bourgeois, R.L. Jernigan, S.C. Szu, E.A. Kabat and T.T. Wu, Biopolymers 18 (1979) 2625.
[26] J. Ramstein, M. Charlier, J.C. Maurizot, A.G. Szabo and C. Helene, Biophys. Biochem. Res. Comm. 88 (1979) 124.